Genetic and biochemical characterization of two new extradiol dioxygenases of *Sphingomonas* sp. strain RW1 and construction of two gene cassettes for biodegradation of dibenzofuran and dioxin

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1 Introduction

During various industrial processes several xenobiotics, e.g. chlorinated aromatic and aliphatic hydrocarbons, occurring as products or by products, are often released into the environment where they accumulate as recalcitrant pollutants, highly toxic for the biosphere. Polychlorinated dibenzofurans (PCDBFs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) (Fig 1) are among the most toxic xenobiotics (Krowfke, 1986). They are generated during combustion of chlorine-containing organic materials or chemical processes, and cause significant public concern because they are ubiquitously present, even in food (Kalantzi *et al*., 2001).

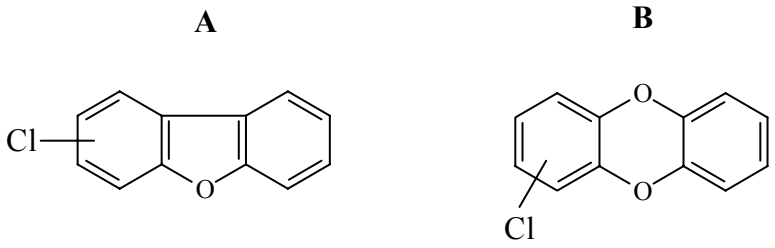


Fig.1.1 Chlorinated dibenzofurans (A) and dibenzo-*p*-dioxins (B)

One possible approach to detoxify the environment from xenobiotics is the utilization of microorganisms which are abundantly present in soils and which are known to be major agents in the mineralization of organic substrates. In the recent years, various microorganisms have been isolated for their capability of transforming or even mineralizing xenobiotic organic compounds (Chaudry and Chapalamadugu, 1991). Microbial consortia capable of mineralizing compounds previously assumed to be recalcitrant have been established in laboratories giving encouraging results for future bioremediation technologies (Arfmann *et al*., 1997). The effective application of microorganisms for the detoxification of the environment requires a detailed knowledge about the relevant catabolic processes and crucial reactions, as well as on factors influencing their efficiency and the development of optimization strategies to overcome critical metabolic steps. The microbial degradation of monocyclic aromatics has been intensively studied (Zylstra and Gibson, 1991) (Sariaslani, 1989). Microbial degradation of bicyclic compounds such as polychlorinated biphenyls (PCB), and naphthalenes and of polycyclic aromatic hydrocarbons (PAHs), has received increasing

attention during the last years. However, studies on the microbial degradation of dibenzo-*p*-dioxins (DDs) and dibenzofurans (DBFs) have been scarce, probably due to the difficulty of isolating microorganisms able to attack those. Furthermore, highly chlorinated DBFs and DDs, especially those that have at least three out of four chlorine atoms in the 2,3,7,8-positions, are extremely toxic (Tyskind *et al*., 1993) and therefore dangerous to be handled in a laboratory. The best documented example of a highly toxic chlorinated DD is the cancerogenic and teratogenic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Zober *et al*., 1998). Large quantities of this compound were discharged into the environment following the tragic accident in Seveso in 1976 during the manufacturing of the herbicide 2,4,5-trichlorophenoxyacetate (Hay, 1979). Until now, no microorganisms able to mineralize highly chlorinated DBFs and DDs have been isolated. However, bacteria able to aerobically mineralize unchlorinated and transform monochlorinated congeners have been isolated in the recent years. As it is known that anaerobic microbial consortia can reductively dehalogenate a wide range of polychlorinated organic compounds including the respective dibenzofurans and dibenzo-*p*-dioxins (Barkovskii and Adriaens, 1996), a promising biodegradation approach could consist of a combination of anaerobic and aerobic microbial activities, i.e. an initial reductive dehalogenation step leading to lower chlorinated congeners followed by aerobic degradation of the carbon skeleton as later discussed. The first organisms being capable to mineralize dibenzofuran have been described by (Strubel *et al*., 1989) and Fortnagel et al. (Fortnagel *et al*., 1989). Both *Terrabacter* sp. DPO1361 (Schmid *et al*., 1997) formerly named Brevibacterium sp. DPO1361 (Strubel *et al*., 1989) and *Sphingomonas* sp. HH69 (Harms *et al*., 1995) formerly named *Pseudomonas* sp. HH69 (Fortnagel *et al*., 1990) converted dibenzofuran to 2,2'.3-trihydroxybiphenyl and salicylic acid indicating a novel angular dioxygenation mechanism at the 4,4a position of dibenzofuran. Similarly, *Staphylococcus auriculans* DBF 63 (Monna *et al*., 1993), recently also reclassified as Terrabacter, (Kasuga *et al*., 2001), and *Terrabacter* sp. DPO360 (Schmid *et al*., 1997), can mineralize dibenzofuran (Schmid *et al*., 1997). In contrast to the strains mentioned above, strain *Sphingomonas* sp. RW1, isolated from the river Elbe (Wittich *et al*., 1992), is capable not only of degrading dibenzofuran, but additionally to mineralize dibenzo-*p*-dioxin, a compound which by other strains is only subject to cometabolism (Monna *et al*., 1993) (Harms *et al*., 1990). Due to its exceptional catabolic characteristics, which include the capability to transform some mono- and dichlorinated congeners such as 1- and 2-chlorodibenzo-*p*-dioxin, 2,3-dichlorodibenzofuran and 2,3-dichlorodibenzo-*p*-dioxin (Wilkes *et al*., 1996) strain

RW1 in the recent years was subject to various physiological, biochemical and genetic studies. The catabolic pathways for dibenzofuran and dibenzo-*p*-dioxin in RW1 have been proposed by Wittich et al. (Wittich *et al*., 1992) and are presented in fig. 1.2. The metabolism of both DBF and DD is initiated by a stereospecific angular dioxygenation of one of the aromatic rings leading to the formation of phenolic hemiacetals which spontaneously rearomatize to give 2,2',3-trihydroxybiphenyl and 2,2',3-trihydroxydiphenyl ether, respectively. The trihydroxylated intermediates are subjected to an extradiol ring cleavage, similar to that described for ring-cleavage of 2,3-dihydroxybiphenyl during the bacterial metabolism of biphenyl (Eltis *et al*., 1993). 2-hydroxy-6-(2-hydroxyphenyl)-6-oxo-hexa-2,4-dienoic acid (2-OH-HOPDA), the ring-cleavage product of 2,2',3-trihydroxybiphenyl (Strubel *et al*., 1991) is subjected to hydrolysis by 2-hydroxy-6-oxo-6-phenylexa-2,4-dienate-type hydrolases resulting in the formation of salicylate and 2-hydroxypenta-2,4-dienoate (Bunz *et al*., 1993) a reaction equivalent to that for hydrolysis of 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoate during biphenyl metabolism. In contrast, the 6-(2-hydroxyphenyl)ester of 2-hydroxymuconic acid, reported as ring-cleavage product of 2,2',3-trihydroxydiphenyl ether was supposed to be subjected to spontaneous hydrolysis, giving rise to catechol and 2-hydroxymuconate (Wittich *et al*., 1992) (Wittich personal communication).Thus, by enzymes of the "upper pathway", dibenzofuran and dioxin are transformed into salicylate and 2-hydroxypenta-2,4-dienoate on one hand and into catechol and 2-hydroxymuconate on the other hand. 2-Hydroxymuconate (Whitman *et al*., 1992) and 2-hydroxypenta-2,4-dienoate (Lian and Chapman, 1993; Lian and Chapman, 1994) are well documented intermediates of the catechol *meta*-cleavage pathway. As a catechol *meta*-cleavage pathway is induced during degradation of both DD and DBF by RW1 (Wittich *et al*., 1992), mineralization of those compounds can be assumed. Beside a catechol *meta*-cleavage pathway, a catechol *ortho*-cleavage pathway is induced during DD degradation. Thus, the intermediate catechol can be degraded via both of those pathways, however, the relative importance of each of those routes cannot be assessed. In case of DBF degradation, both catechol *meta*-cleavage pathway as well as gentisate pathway are obviously induced. However, there are no data available, showing whether salicylate in RW1 is degraded by a salicylate 1-hydrolase and thus via catechol or by a salicylate 5-hydrolase and thus via gentisate.

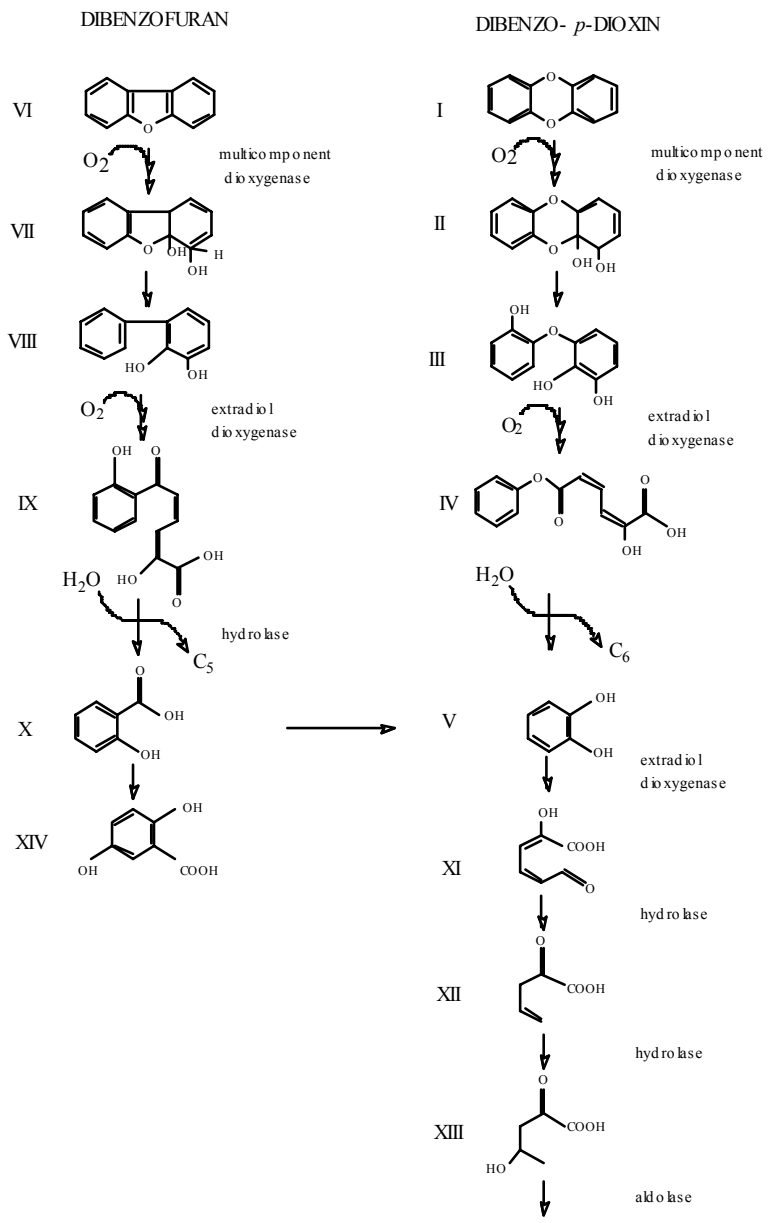


Fig.1.2: Proposed catabolic pathway for dibenzofuran and dibenzo-*p*-dioxin in *Sphingomonas* sp. strain RW1. (I) dibenzo-*p*-dioxin (II) dibenzo-*p*-dioxin-cis-dihydrodiol (III) 2,2',3-trihydroxybiphenyl ether (IV) 2-hydroxy-6-oxo-6-(2-hydroxyphenoxy)-hexa-2,4-dienoate, (V) catechol, (C6) 2-hydroxy-cis,cis-muconate, (VI) dibenzofuran, (VII) dibenzofuran-cis-dihydrodiol, (VIII) 2,2',3-trihydroxybiphenyl, (IX) 2-hydroxy-6-oxo-6-(2-hydroxyphenyl-hexa-2,4-dienoate, (X) salycilate, (C5) 2-hydroxypentadienoate semialdehyde, (XII) 2-hydroxypenta-2,4-dienoate, (XIII) 4-hydroxy-2-oxovalerate and (XIV) gentisate. Unstable compounds which spontaneously transform to other products are indicated in brackets. (Armengaud and Timmis, 1997a)

1.1 Biochemical Characterization of the dioxin dioxygenase multicomponent complex

The degradation of aromatic compounds is usually initiated by ring-activating reactions. In case of dioxygenolytic activation, the aromatic ring is attacked by the incorporation of two hydroxyl groups in *ortho* position to one another (Dagley, 1978). The *cis*-dihydrodiols formed thereby are then rearomatized by *cis*-dihhydrodiol dehydrogenases. If the aromatic ring is substituted, the dioxygenation reaction can occur either on two adjacent unsubstituted carbon atoms or directly at a substituted and its neighbouring carbon atom. In the latter case, the reaction can be concomitant with the spontaneous elimination of the substituent, when the substituent can be eliminated as an anion. The formation of unstable dihydrodiol analogues has been reported during the degradation of haloaromatics such as 2-chlorobenzoate (Engesser and Schulte, 1989), 1,2,4,5-tetrachlorobenzene (Beil *et al*., 1998b) or 2,2'-dichlorobiphenyl or sulfoaromatics (Haddock *et al*., 1995.). Due to spontaneous rearomatization, a dehydrogenase is nor required for degradation. Similar unstable dihydrodiol analogues, hemiacetals, will be formed during angular dioxygenation of biarylethers. Spontaneous rearomatization by loss of a phenolate group, which is simultaneously the cleavage of the former ether bond, results in the formation of two monocyclic aromatics from simple biarylethers (Engesser *et al*., 1990; Schmidt *et al*., 1992) or of dihydroxybiphenyl derivatives from dibenzofuran and dibenzo-*p*-dioxin, respectively. Ring-hydroxylating dioxygenases require oxygen, ferrous iron (Fe2+) and reduced pyridine nucleotides for catalysis. They are soluble, multicomponent enzyme systems comprising two or three separate proteins. In principle, these enzymes consist of a electron transport chain that channels the electrons from NAD(P)H to the catalytic terminal oxygenase component where substrate transformation takes place. The terminal oxygenase component usually contains a Rieske (2Fe-2S)-cluster and mononuclear iron. Together with the respective monooxygenases those enzymes are termed Rieske non-heme iron oxygenase (Gibson and Parales, 2000). The electron transport chain is composed of, either an iron-sulphur flavoprotein reductase, or two separate proteins, a flavoprotein reductase and an iron-sulphur ferredoxin.

The structure of ring-hydroxylating oxygenases differs considerably. The variation in the number and size of constituent compounds and in the nature of redox centers has been used to classify these enzymes into three groups as Class I, II, III (Batie and Kamin, 1986) (Batie *et al*., 1992). In the reductases of class I and Class III

dioxygenases both a flavin chromophor (FAD in Class IA and III or FMN in Class IB) and a plant-type [2F-2S] iron-sulphur cluster are present. Class III enzymes additionally contain a separate ferredoxin component carrier with a Rieske type [2F-2S] iron-sulphur cluster that serves as an intermediate electron-transfer. Reductases of Class II enzymes only contain FAD as prosthetic group and transfer the electrons via a separate ferredoxin with either a plant-type (ClassIIA) or Rieske-type (ClassIIB) [2F-2S] iron-sulphur cluster to the terminal oxygenase. However, this system of classification is based mainly on the electron transport components and did not take into account information on the oxygenase itself. For example, 2-oxo-1,2-dihydroquinoline monooxygenase (Rosche *et al*., 1995) catalyzing a monooxygenase reaction and benzoate dioxygenase (Neidle *et al*., 1991) catalyzing a dioxygenase reaction are assigned to Class IB, as both systems have only an iron-flavoprotein reductase. In addition, the quinoline oxygenase component has a $(\alpha)6$ composition, whereas benzoate dioxygenase have a $(\alpha\beta)3$ subunit composition. As the catalytic α-subunit components of all Rieske non-heme iron oxygenases on one hand define the substrate specificity of the enzyme complex (Beil *et al*., 1998a) (Mondello *et al*., 1997) and on the other hand are related to each other, Gibson and Parales (Gibson and Parales, 2000) proposed the oxygenase components to be considered as superfamily, which consists of clearly identifiable families.

The stereospecific angular dioxygenation of dibenzofuran and dibenzo-*p*-dioxin in *Sphingomonas* sp. RW1 is carried out by the dibenzofuran 4,4a-dioxygenase (Bunz and Cook, 1993). The subunits constituting the electron supply system and the oxygenase itself were purified and biochemically characterized by Bünz and Cook (1993). The oxygenase was supposed to be a $\alpha2\beta2$ heterodimer with subunit molecular weights of 45 kDa (large α-subunit) and 23 kDa (small β subunit), respectively. UV-visible absorption spectroscopy indicated the presence in the large subunit of a Rieske-type 2Fe-2S cluster usually contained in oxygenase subunits of ring-activating oxygenases. However the N-terminal sequence of the a subunit of the dioxin dioxygenase did not exhibit any significant similarity with other previously described dioxygenases. In vitro the dibenzofuran 4,4a-dioxygenase consisting of the oxygenase and the respective electron transport chain was able to transform both dibenzofuran (into 2,2',3-trihydroxybiphenyl) and dibenzo-*p*-dioxin (into 2,2',3-trihydroxybiphenyl ether) by introduction of two atoms of oxygen from one single molecule of O_2. The two oxygen atoms (fig. 1.2) are added to a pair of vicinal carbons one of which is involved in one of

the bridges between the two aromatic rings (Bunz and Cook, 1993). The enzyme could additionally transform biphenyl, xanthene, fluoren-9-one and dibenzothiophene.

Two isofunctional, monomeric flavoproteins RedA1 and RedA2 capable of transferring electrons form NADH to a ferredoxin, which finally transported electrons to the oxygenase component, could be purified (Bunz and Cook, 1993). It was speculated that RedA2 constitutes the main reductase component of the electron transfer chain. Both proteins contained a labile flavine adenine dinucleotide cofactor but no iron-sulphur cluster, characteristica which clearly distinguishes them from reductases associated with Class III ring hydroxylating dioxygenases, such as naphthalene dioxygenase (Ensley *et al*., 1982), and from those associated with Class IA and Class 1B enzymes (Batie *et al*., 1992). Again, no significant similarity was found between their N-terminal sequence and those of other known flavoproteins. The electron transfer system associated with the dioxin dioxygenase therefore appears unusual.

The second component of the redox chain is a small ferredoxin, of 12 kDa in size. Protein sequence data showed that the ferredoxin resembles putidaredoxin (Armengaud and Timmis, 1997b) containing a plant type iron-sulphur cluster coordinated by four cysteine residues and indicated the ferredoxin to be unrelated to the Fe-S proteins which usually function as electron donor to ClassIIB ring hydroxylating dioxygenases and which contain a Rieske-type 2Fe-2S cluster coordinated by two cysteins and two histidines. It has therefore been proposed that the new multiple component dibenzofuran 4,4a-dioxygenase of *Sphingomonas* RW1, according to the classification scheme of Batie et al. (Batie *et al*., 1992) belongs to ClassIIA of ring hydroxylating dioxygenases. The dioxin dioxygenase would then have been, beside the pyrazon dioxygenase described by Sauber et al. (Sauber *et al*., 1977), the second representative of this class. Another dioxygenase, the carbazole 1,9a-dioxygenase from *Pseudomonas* sp. strain CA10, has been recently characterized (Nojiri *et al*., 1999), which is able to catalyze an angular dioxygenation of several substrates in the same way as the dibenzofuran 4,4a-dioxygenase of RW1, and also a mono-oxygenation as well as a *cis* dihydroxylation. The multiple capabilities of this enzyme and the broad substrate specificity made it interesting to attempt the purification and crystallization of the protein, which will surely reveal an interesting structural feature and a biochemistry maybe similar to that of RW1 dibenzofuran dioxygenase.

1.2 Genetic characterization of the enzymes constituting the dioxin dioxygenase complex

The gene encoding the 2Fe-2S ferredoxin, *fdx1*, was amplified by PCR from a genomic library (Armengaud and Timmis, 1997b). The purified ferredoxin contained a putidaredoxin-type 2Fe-2S cluster. Fdx1 shares significant homology to several plant-type 2Fe-2S ferredoxins involved in electron transfer to bacterial cytochrome P450 containing monooxygenases. Those ferredoxins are phylogenetically unrelated to Rieske-type ferredoxins such as BedC1 and TodB which were up to now usually described to function in electron transfer to ring-hydroxylating dioxygenases (Erickson and Mondello, 1992; Morrice *et al*., 1988; Zylstra and Gibson, 1991). Fdx1 constitutes therefore an atypical electron transfer system for a multicomponent dioxygenase (Sauber *et al*., 1977). It has been clearly shown that this ferredoxin is able to transfer electrons to the dioxin dioxygenase in a reconstituted system (Armengaud *et al*., 1998; Bunz and Cook, 1993). The dioxin dioxygenase was active with both substrates dibenzofuran and dioxin, giving as a reaction product 2,2',3-trihydroxybiphenyl and 2,2',3-trihydroxybiphenyl ether. On the other side no activity was observed when the dioxin dioxygenase was coexpressed with a class IIB electron-transfer system recruited from the biphenyl gene cluster of LB400 (Armengaud *et al*., 1998). These biochemical data confirmed that the dibenzofuran dioxygenase of RW1 belongs to the multi-component dioxygenase class IIA. The sequence analysis of the DNA regions upstream and downstream of *fdx1*, revealed this gene surprisingly not to be clustered with either known oxido-reductases nor the genes encoding the α- and β-subunits of the dioxygenase itself. The gene *fdx1* is, in contrast, located between two ORFs, whose sequences are related to that of a bifunctional protein which acts as a decarboxylase and an isomerase in the 4-hydroxyphenylacetic acid catabolic pathway of *E. coli* strain (Prieto *et al*., 1996). In the dibenzofuran/dioxin degradative pathway proposed for *Sphingomonas* sp. strain RW1 (Wittich *et al*., 1992) a role of such activities is not evident and it is still not clear whether these proteins could play a function for dioxin degradation.

Additionally a gene encoding a protein with sequence similarities to glutathion-*S*-transferases (GST) was identified downstream of the *fdx1* gene. Genes encoding several classes of GST enzymes have been found to be broadly distributed among PAH-degrading microorganisms (Bartels *et al*., 1999) (Lloyd-Jones and Lau, 1997) (Shin *et al*., 1997) (Wang *et al*., 1994). The role played by GSTs in PAH degradation is still

unclear. In the case of BphK GST of *Burkholderia* sp. strain LB400, experimental evidences showed the GST enzyme not to be necessary for growth on biphenyl as a substrate (Bartels *et al*., 1999). However, because of the broad distribution of such enzymes and their high degree of sequence identity, it has been suggested they might have a function improving and supporting PAH degradation under specific reaction conditions. Also the gene encoding the flavoprotein, reductase RedA2, has been amplified by PCR from the genomic library of RW1 (Armengaud and Timmis, 1998). From the nucleotide sequence as well as from the biochemical data obtained with the recombinant form of this reductase, it is evident that this flavoprotein is similar to class-I cytochrome P450-type reductases (Munro and Lindsay, 1996; Unger *et al*., 1986). Like *fdx1*, *redA2* is not directly linked to any other gene encoding for dibenzofuran dioxygenase subunits, a ferredoxin or to oxygenase related genes.

The *dxnA1* and *dxnA2* cistrons, encoding the large and small subunits of the dioxin dioxygenase, respectively, were also amplified from a genomic library by PCR (Armengaud *et al*., 1998). Since the genes of multicomponent dioxygenase systems were previously nearly exclusively found to be clustered, initially a PCR product was expected, which comprises both the genes encoding for the dioxin dioxygenase and that ones for the ferredoxin and/or reductase. However, like *fdx1* and RedA2, *dxnA1* and *dxnA2* were not linked to any other gene necessary for encoding a functional dioxygenase. They were, nevertheless, coamplified with the gene encoding a HOPDA hydrolase, supposed to catalyze the third enzyme step in the dibenzofuran degradative pathway. The overall identity of the DnxA1 sequence with its counterparts in classIIB and class III dioxygenases is relatively low (40%) but clearly shows a phylogenetic relationship with three-component dioxygenases. Even though the homology research revealed DxnA1 not to be a member of previously described major clusters of Rieske type non-heme iron α-subunits of oxygenases, the major motifs common to this family, such as the environment of the Rieske-type 2Fe-2S cluster and the FeII binding site, are conserved compared to other three-component dioxygenases (Jiang *et al*., 1996). Interestingly in the FeII binding site of the large subunit a histidine residue is found at position 202 in place of a usual phenylalanine and a leucine residue at position 207 was found instead of a usual tyrosine. The two residues might play a critical role in the peculiar regiospecificity of the dioxin dioxygenase. The *dxnA2* ORF codes for a polypeptide exhibiting 39% identity to the β subunits of the three-component

dioxygenases. Therefore DxnA1 and DxnA2 have the same phylogenetic distance from their class IIB counterparts.

1.3 Biochemical and Genetic characterization of the *meta*-cleavage enzyme DbfB

The ring-cleavage of 2,2',3-trihydroxybiphenyl and the 2,2',3-trihydroxybiphenyl ether formed from dibenzofuran and dioxin, respectively, is catalyzed by an extradiol or *meta*-cleavage dioxygenase termed DbfB or trihydroxybiphenyl dioxygenase (Fig.1.2). Its structural gene designed *dbfB* has been cloned from a cosmid expression library of RW1 using a functional colorimetric assay, and sequenced (Happe *et al*., 1993). The gene *dbfB* has been localized, 4.5 Kb upstream of *dxnA1A2* in the RW1 genome and opposite oriented (Armengaud *et al*., 1998). The deduced amino acid sequence reveals the enzyme to be a typical extradiol dioxygenase, as recently shown by a structure-validated alignment (Eltis and Bolin, 1996; Han *et al*., 1995). Extradiol dioxygenases are a family of ferrous iron-containing enzymes that mediate aromatic ring cleavage in a wide variety of aromatic catabolic pathways, including those of naphthalene, benzene, biphenyl, and toluene (Harayama *et al*., 1992). The pivotal role of these enzymes has been revealed by genetic studies on the TOL pathway, which indicated that its extradiol dioxygenase is a major determinant of substrate specificity of this pathway (Ramos *et al*., 1987). Some specific reaction intermediates like 3-chlorocatechol, formed during the catabolism of a number of chlorinated aromatics, can severely inhibit the activity of these enzymes. This phenomenon represents a critical aspect in attempting the biodegradation of recalcitrant compounds. As an example, 1-chlorodibenzo-*p*-dioxin is transformed by RW1 into 3-chlorocatechol. Attack of trihydroxybiphenyl dioxygenase on this compound obviously leads to the formation of a reactive acylchloride, as described by Bartels et al. (Bartels *et al*., 1984), irreversibly activating this enzyme. As a consequence, activity against trihydroxybiphenyl is destroyed (Wilkes *et al*., 1996).
After hyperexpression of *dbfB* in *E. coli*, the resulting protein has been aerobically purified and characterized (Happe *et al*., 1993). Interestingly DbfB resulted to be monomeric. Although other monomeric dioxygenases have been reported (Block and Lingens, 1992), extradiol dioxygenases are generally either tetrameric, such as the catechol 2,3-dioxygenase (Nakai *et al*., 1983; Wallis and Chapman, 1990) or octameric, such as the 2,3-DHB 1,2-dioxygenase (Eltis *et al*., 1993; Furukawa and Arimura, 1987; Taira *et al*., 1988). The significance of the difference in quaternary structure is not clear.

The best substrates of DbfB were 2,2',3-trihydroxybiphenyl and 2,3-dihydroxybiphenyl, whereas monocyclic catechols such as 4-methylcatechol and catechol were only poorly transformed. This substrate spectrum places DbfB, in accordance with sequence similarities, into the subfamily of extradiol dioxygeneses with high activity against bicyclic compounds (Eltis and Bolin, 1996). Like various other extradiol dioxygenase (Eltis *et al*., 1993), DbfB was shown to be subject to substrate inhibition at high concentration of substrate (Happe *et al*., 1993). In contrast to di- and trihydroxybiphenyl, only small activity was observed with trihydroxybiphenyl ether, the intermediate of DD degradation, as a substrate. Moreover, incubation with this substrate resulted in a rapid decline of the observed activity. Since RW1 is able to grow on DD as sole carbon and no major problems during ring-cleavage of intermediates were observed in those growth studies (Wittich *et al*., 1992), the question arises how in vivo trihydroxybiphenyl ether transformation is performed and whether another *meta*-cleavage dioxygenase besides DbfB, plays a major role in the DD degradation pathway by RW1. In this work we addressed this question with experiments oriented to better understand this critical reaction step in the DD catabolic pathway.

Sphingomonas sp. RW1 has been shown to contain, besides DbfB, additional *meta*-cleavage enzymes (Happe *et al*., 1993) (Bunz and Cook, 1993) one of which, designed Edo2, was analyzed in detail in the present study. The presence of multiple *meta*-cleavage enzymes is not an exception to RW1 but has recently been shown in various aromatic compounds degrading bacteria. The dibenzofuran degrader, *Terrabacter* sp. Strain DPO 360, contains three distinct extradiol dioxygenases (Schmid *et al*., 1997) in the same way as *Rhodococcus globerulos* P6, one PCB-degrader, (Asturias and Timmis, 1993). The reason for the multiplicity of such isoenzymes has not yet been definitely explained.

1.4 Biochemical and genetic characterization of RW1 hydrolases

During the purification form dibenzofuran grown cells of RW1 of the peptides constituting the multicomponent dioxin dioxygenase, two hydrolases namely H1, later on *dxnB*, and H2 were identified and characterized (Bunz *et al*., 1993) (Armengaud *et al*., 1998). The gene *dxnB* encoding the hydrolase H1 could be coamplified with *dxnA1A2* and found to be localized precisely 270 bp downstream of *dxnA2*. The N-terminal sequences of the monomeric hydrolases H1 and H2 revealed 50% homology.

Both enzymes could be shown to catalyze the third step in the dibenzofuran degradative pathway, i.e. the hydrolysis of 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoate (HOHPDA) into salicylate and 2-hydroxypenta-2,4-dienoate. They were also active against 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA), the ring-cleavage product of dihydroxybiphenyl, forming benzoate and 2-hydroxypenta-2,4-dienoate. However neither benzoate nor salicylate were produced in stoichiometric amounts. In case of 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoate (HOHPDA) turnover, this can easily be explained by the instability of the substrate which is subject to a spontaneous intramolecular Michael addition (Fortnagel *et al*., 1990) (Strubel *et al*., 1991) (Wittich *et al*., 1992) (Kohler *et al*., 1993) resulting in the formation of 3-(chroman-4-on-2-yl)pyruvate. This observation is not limited to enzymatic reactions in vitro. Formation of chromanone derivatives from HOHPDA has also been reported to occur in growing cells, showing the hydrolysis to be a critical step in dibenzofuran degradation, with the need of a highly active hydrolase with high affinity for the substrate to overcome spontaneous reactions giving rise to dead-end metabolites.

1.5 Overview of the genetic organization for dibenzofuran and dibenzo-*p*-dioxin catabolic pathways in RW1

From the sequencing analysis, the genes coding for the enzymes involved in DD and DF degradation appeared to be dispersed on the chromosome. In fig. 1.3 an overview of the genetic organization is presented. Several loci have been identified (Armengaud and Timmis, 1997a), some of which have been already previously described. Locus A is associated to genes *dxnA1A2*, *dbfB* and *dxnB* encoding respectively the α and β subunits of the dibenzofuran 4,4a-dioxygenase, the *meta*-cleavage dioxygenase DbfB and the H1 hydrolase. Genes *fdx1* and *redA2* encoding for ferredoxin and reductase associated with RW1 dibenzofuran dioxygenase, are carried by respectively locus B and C. Locus D has been identified by hybridizing the cosmidic library of RW1 with a probe obtained by mean of two primers, designed on the basis of DxnA1 N-terminal and of conserved motives in class IIB dioxygenases. Those primers, originally meant to amplify *dxnA1A2*, led to the amplification of a short fragment, carrying an ORF encoding a polypeptide showing identity to another α-subunit ring-hydroxylating dioxygenase. By analyzing the surrounding sequence of *edo2* gene, carried by Locus E, additional ORFs

were identified, sharing homology with α and β subunits of ring-hydroxylating dioxygenases and indicated in fig.1.3 as ORFs G1, G5, G6. Locus E, furthermore, was found to present several degradative genes including that of a monooxygenase specified by ORF G4. The deduced polypeptide sequence of ORF G4 exhibited significant identity with several flavin-containing monooxygenases from different sources. Between *edo2* (ORF G2) and ORF G4, a short ORF (106 aa) has similarities to Rieske-type 2F-2S ferredoxins associated with class IIB and III ring-hydroxylating dioxygenases. ORF G7, downstream of G4, specifies for a polypeptide homologous to HOPDA hydrolases. This cistron resembles that one of locus A where the genes *dxnA1A2* and *dbfB*, *dxnB*, and *dxnC* are located. *dxnC* is the first of ten ORFs identified downstream *dxnB* and apparently organised in a compact operon as recently described (Armengaud *et al*., 1999). The first three ORFs of the locus, *dxnC*, *ORF2*, and *fdx3*, specify a protein with a low homology to bacterial siderophore receptors, a polypeptide representing no significant homology to known proteins, and a putative ferredoxin, respectively. In in vitro assays Fdx3 could be reduced by RedA2. In vivo coexpression of *fdx3* and *redA2* with *dxnA1A2* confirmed that Fdx3 can serve as an electron donor for the dioxin dioxygenase also. The midpoint redox potential of this ferredoxin is similar to that exhibited by Fdx1. The gene *fdx3* if followed by gene *dxnD* which encodes a 69-kDa phenol monooxygenase-like protein with activity for the turnover of 4-hydroxysalicylate, and by *dxnE* encoding for a 37-kDa protein whose sequence and activity are similar to those of known maleylacetate reductases. The following *dxnF*, encodes a 33-kDa intradiol dioxygenase which efficiently cleaves hydroxyquinol, yielding maleylacetate, the ketoform of 3-hydroxy-cis,cis-muconate. The heteromeric protein encoded by the following *dxnGH* is a 3-oxoadipate succinyl coenzyme A (succinyl-CoA) transferase, whereas *dxnI* (Armengaud *et al*., 1999) specifies a protein exhibiting marked homology to acetyl-CoA acetyltransferases (thiolases). The last ORF of the operon codes for a putative transposase. *dxnD*, *dxnF*, *dxnE*, *dxnGH*, and *dxnI* thus form a complete 4-hydroxysalicylate/hydroxyquinol degradative pathway. Summarizing the genetic diversity of *Sphingomonas* sp. RW1, 4 different α-subunits of ring-hydroxylating dioxygenase, two β-subunits, two reductases, three ferredoxins, three *meta*-cleavage enzymes have been found until now. Furthermore, as previously described, two different hydrolases, H1 and H2, could be isolated. A third hydrolase H3 was identified in the present study. The *H3* structural gene was found to be clustered with that one of RW1 *meta*-cleavage dioxygenase Edo 3, also identified and described

for the first time in this study. *H3* and *edo2* genes should be associated to a new locus. A similar genetic organization to that one on RW1, where genes encoding for enzymes involved in a same degradation pathway are found to be dispersed among several gene cluster, has been described by M. Romine et al. in case of *Sphingomonas aromaticivorans* F199 (Romine *et al*., 1999). Also F119 contains several oxygenase subunits which appears to be characteristic of *Sphingomonas* sp. It has been suggested that the respective gene products could randomly combine to give hybrid aromatic oxygenases. It is unclear why *Sphingomonas* sp. strain RW1 presents several groups of isoenzymes and whether, for instance, they play a role in different metabolic pathways. With this work we have started to address this question, especially concerning the three *meta*-cleavage enzymes.

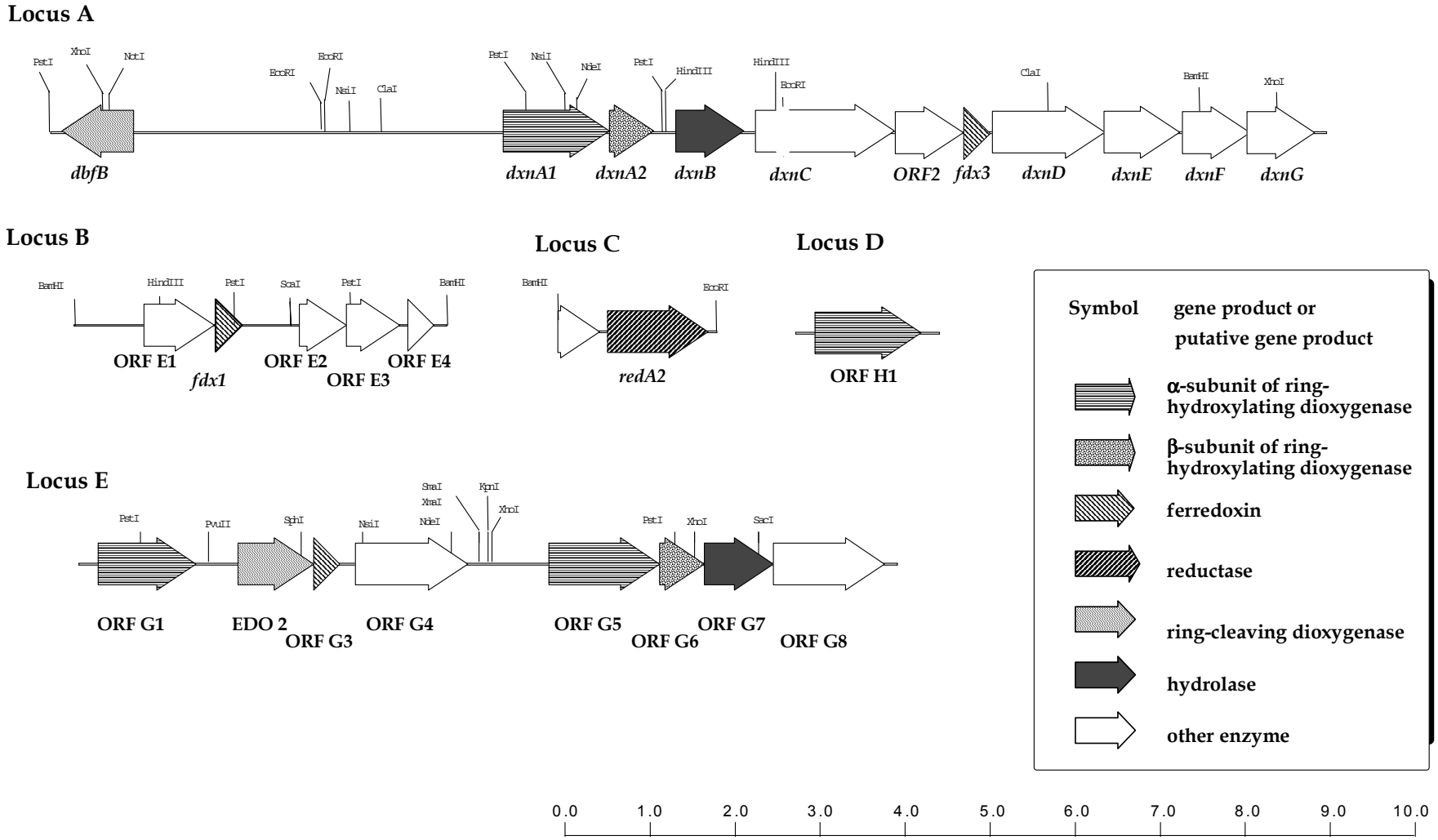


Fig. 1.3: Genetic organization of RW1 loci as described in (Armengaud *et al*., 1998)

1.6 Goal

The detailed investigation on a genetic and biochemical level of dioxin and dibenzofuran catabolism in *Sphingomonas* sp. strain RW1 is necessary to clarify the limitations and possible bottlenecks of the pathways, especially when confronted with chlorinated congeners, to identify the enzymes and respective genes necessary for degradation and finally to develop strategies for the optimization of bacteria to detoxify in the environment highly recalcitrant chlorinated dibenzofurans and dioxins.

The usefulness of this strategy can be seen in efforts to obtain biocatalysts for the clean-up of sites contaminated with PCBs. Various biphenyl degraders have been analyzed in detail for the degradative route, and the genes and the gene products involved in the transformation of different PCB congeners have been identified. The range of PCBs transformable was show to depend mainly on the specificity of the initial dioxygenase (Erickson and Mondello, 1993; Mondello *et al*., 1997). The amino acids defining the substrate specificity could be defined and enzyme variants capable to transform a range of PCB congeners, previously not metabolizable by a single organism, could be engineered (Hrywna *et al*., 1999; Kumamaru *et al*., 1998). Natural isolates, when confronted with PCBs, in the best case, transform them into chlorobenzoates, as degradative pathway for chlorobenzoates in those strains is usually missing. Misrouting of excreted chlorobenzoates by the natural microflora can result in the formation of highly toxic intermediates, thus having a severe negative effect on the performance of PCB transforming organisms in environments contaminated with PCBs (Blasco *et al*., 1995). In order to achieve complete mineralization and thus avoiding substrate misrouting, either cocultures of strains transforming PCBs and those capable of mineralizing chlorobenzoates (Park *et al*., 1999) were applied, or alternatively, organisms were engineered to contain a complete metabolic route (Hrywna *et al*., 1999; Reineke, 1998).

In contrast to the knowledge of biphenyl degradation and PCB transformation, there is only limited information on dioxin/dibenzofuran degradation and transformation of chlorinated congeners. The best described organism, *Sphingomonas* sp. RW1 can grow on dioxin and dibenzofuran as sole carbon source, but not on chlorinated dioxins and dibenzofurans which is apparently due to at least three reasons: the limited substrate range of the intial dioxin dioxygenase, the formation of dead-end products and the formation of toxic intermediates. Inhibition of extradiol dioxygenases by intermediarly formed 3-chlorocatechol was observed during 1-chloro-dibenzo-*p*-dioxin conversion by

resting cells of RW1. 3-chlorocatechol has often been reported to inhibit extradiol ring-cleavage dioxygenases (Bartels *et al*., 1984; Klecka and Gibson, 1981; Wasserfallen *et al*., 1991). The best studied example is the catechol 2,3-dioxygenase of *Pseudomonas putida* mt-2 where the mechanism of inhibition was investigated (Bartels *et al*., 1984). The deactivation agent was supposed to be the ring-cleavage product 5-chloroformyl-2-hydroxypenta-2,4-dienoic acid, an acylchloride, formed by 2,3-clevage of 3-chlorocatechol which irreversibly inactivates the ring-cleavage dioxygenase.

Recently a novel extradiol dioxygenase has been purified from the bacterial strain *Pseudomonas putida* GJ31 (Kaschabek *et al*., 1998). This strain is able to degrade the chlorobenzene via 3- chlorocatechol and a *meta*-cleavage pathway (Mars *et al*., 1997), thus indicating that the enzyme is not subject to suicide inactivation as reported for the enzyme of strain mt-2. Actually, the enzyme was capable of a high rate turnover of 3-chlorocatecol and 2-hydroxymuconate was found to be the *meta*-cleavage product. It can be reasoned that the initial acylchloride ring-cleavage product is subject to a rapid hydrolysis by the GJ31 enzyme whereas nucleophilic groups of the mt-2 enzyme react rapidly with the acylchloride to give inactive acylated enzyme derivatives. An alternative mode to overcome suicide inactivation has been reported by Heiss et al. (Heiss *et al*., 1995). A 2,3-dihydroxybiphenyl dioxygenase from the naphtalenesulfonate degrading *Sphingomonas* sp. strain BN6 cleaves 3-chlorocatechol between the C1 and C6 carbon thus resulting in the formation of 3-chloro-2-hydroxymuconic semialdehyde, thereby avoiding the formation of an acylchloride. However despite the evidence that chlorocatechols can be degraded after *meta*-cleavage (Arensdorf and Focht, 1994), the dominant metabolic route for chlorocatechols degradation is via a chlorocatechol *ortho*-cleavage pathway. There are no indications that RW1 exhibits respective genes or activities.

Whereas chlorinated dibenzo-*p*-dioxins, in case one aromatic ring is degraded, are transformed into catechols, the respective salicylates are the products when chlorodibenzofurans are transformable by enzymes of the dibenzofuran upper pathway of RW1. 3-,4-, and 5-chlorosalycilate, respectively, have been observed to accumulate as dead end products when RW1 is exposed to 4-chloro-, 3-chloro, or 2-chlorodibenzofuran (Wilkes *et al*., 1996; Wittich, 1997). One strategy to achieve mineralization of chlorodibenzofurans can be the application of mixed cultures, consisting of a chlorodibenzofuran transforming organism, which should be capable to grow at the expense of one aromatic ring during this process, and a chlorosalicylate degrader. Some chlorosalicylate degrading organisms have been reported to date,

usually recruiting a salicylate 1-hydroxylase capable of transforming chlorosalicylates into chlorocatechols, followed by a chlorocatechol *ortho*-cleavage pathway resulting in transformation of chlorocatechols into Krebs cycle intermediates. However, some deviations of this route have been reported, such as *Pseudomonas* sp. RW1, which do not contain a chlorocatechol *ortho*-cleavage pathway and probably mineralize 4-chlorosalicylate via 4-chlorocatechol and classical catechol *ortho*-cleavage pathway with protoanemonin as intermediate, or *Sphingomonas* RW5, which seems to degrade 3,6-dichlorosalicylate via dichlorogentisate (Werwath *et al*., 1998).

Actually 4-chloro-dibenzofuran could be mineralized by a coculture consisting of RW1, producing 3-chlorosalicylate, and the 3-chlorosalicylate degrader *Burkholderia* sp. strain JWS (Arfmann *et al*., 1997). Another example of a coculture for mineralization of chlorodibenzofurans is given by *Sphingomonas* sp. strain RW16 and *Pseudomonas putida* RW10 capable together of mineralization of 2- and 3-chlorodibenzofuran. *Sphingomonas* RW16 converts the two substrates into 5-chloro- and 4-chlorosalicylate respectively, which then are degraded by *Pseudomonas putida* RW10 to 4-chlorocatechol and then further mineralized by a protoanemonin pathway (Wittich, 1999). The bioavailability of chlorinated DBFs and DDs decreases with increasing chlorine substitution. Efficient degradation by co-oxidation therefore seems to be restricted to several dichlorinated congeners of DD and DBF. At least the 2,8-DCDF is dioxygenated at low rate by resting cells of *Sphingomonas* RW1 under concomitant release of HCl to a dead end product. Mixed cultures seem to be therefore a feasible approach for biodegradation of low halogenated compounds. An alternative to the use of mixed cultures is the application of genetic engineering techniques to enhance the degradative capabilities of a single organism. By this strategy, the intermediate excretion of intermediates, which can result in misrouting to unwanted products by other microorganisms, can be avoided. Furthermore, various different changes can be performed in a single strain by genetic techniques to optimize the performance.

Mineralization of chlorinated dioxins necessitates a functional dioxin upper pathway and a pathway for chlorocatechol degradation. Coculture degradation needs the accumulation of chlorocatechols by a dioxin degrading organism which should be taken up by the second member of the consortium. However catechols and especially chlorocatechols exert their deleterious effects even at low concentration, and are supposed to be itself toxic. Thus coculture would necessitate a very effective consortium. In contrast, combination of the necessary catabolic modules in one strain could, at least theoretically, prevent any significant intracellular accumulation of toxic

intermediates.In order to create organisms capable to mineralize chlorodioxins and chorodibenzofurans, a major goal of the present study was the development of a gene cassette carrying all genes for the DD degradation upper pathway from *Sphingomonas* RW1. Such cassette could be used to enlarge the catabolic capabilities of chlorosalycilate and chlorocatechol degrading microorganisms able to include chlorinated DDs and DBFs. The new degraders could be useful for detoxification of these highly recalcitrant and toxic xenobiotics.

2 Materials and methods

2.1 Instrumentation

Agitator	SM25Edmund Bühler
Anaerobic chamber	Coy
Autoclave	Tecnomara
Balance	PM460 and AE260, Mettler
Bench centrifuges	Biofuge Haereus Sepathec, Eppendorf centrifuge 5415
Cell incubators	Heraeus B5060, EK002
Centrifuges	Sorvall Instruments RC5, RC3
Computer	Macintosh and IBM
Computer programs	Microsoft Word, Deneba Canvas, CSC ChemDraw Pro, KaleidaGraph, GeneWork, Phylip pakage
Drying machine	Heraeus 5042
Electrophoresis chambers	BRL Horizon 58 and 5320
FPLC	Controller LCC-500, pump P500, fraction collector 100, everything by Pharmacia
French press	SLM Amico
Heating bloc	Eppendorf Thermostat 5320
HPLC system,	Diode array-detector SPD-10M10A, LC-10AD pump, DGU-3A degaser, FCV10 AC valve all by Pharmacia
MilliQ water purifier	Millipore Milli-Q System
Oxygen electrode	Bachofer
PCR thermocycler	Perkin Elmer (GeneAmp PCR system 2400)
pH-meter	Schott CG804
Powersupply	Bachofer
Protein gels chamber	Bio-Rad Mini Protein II Cell
Spectrophotometer	Beckmann DU-70, Shimadzu UV-2100, Hitachi U 2000
Ultracentrifuge	Sorvall Instruments Combi

Vacuum centrifuge	Savant DNA Speed Vac DNA A110
Vortex	Janke&LKunkel IKA VF2

2.2 Chemicals and reagents

Most chemicals used in this study were obtained from Sigma and Aldrich, and were of the highest grade available. 2,3-Dihydroxybiphenyl (2,3-DHB) for enzymatic tests was obtained from Wako Chemicals GmbH. 2,2',3-trihydroxybiphenyl ether and 2,2',3-trihydroxybiphenyl were gently synthesized by Rolf Wittich, Jean Armengaud and D. Pieper. 2-Pyrone-6-carboxylate was kindly provided by W. Reineke and used to synthesize 2-hydroxymuconic acid as previously described (Kaschabek *et al*., 1998). Oligonucleotide primers were obtained desalted from Gibco BRL and used without further purification. Restriction enzymes and reagents for genetic procedures were purchased from New England Biolabs, Boehringer Mannheim, Promega, United States Biochemicals and Sigma.

2.2.1 Anaerobic preparation of a 50 mM 2,2',3-trihydroxybiphenyl-ether solution

An amount of 11.6 mg purified 2,2',3-trihydroxybiphenyl-ether crystals was weighted and transferred into a Duran 2 ml glass sealed with a caucciu cap and filled up with N_2. Crystals were dissolved inside of the anaerobic chamber with 1 ml methanol previously equilibrated for several hours under a N_2 stream.

2.3 Bacterial strains and plasmids

All genes analyzed in this study originate from the genomic library of *Sphingomonas* sp. strain RW1 (H. Poth). Strain RW1 was isolated by R. Wittich (Wittich *et al*., 1992) for its capability to grow with dibenzo-*p*-dioxin and dibenzofuran as sole carbon sources. *E. coli* strains used in this study are listed in tab. 2.1.

Table 2.1: *E. coli* strains used in the present study

Strain	**Reference**
DH5α	(Hanahan, 1983)
DH10B	(Calvin and Hanawalt, 1988)
BL21(DE3)[LysS]	(Studier and Moffatt, 1986)

Plasmids used in this study are summarised in table 2.2.

Table 2.2: Plasmids used in the present study

Plasmids	**Properties**	**Reference or producer**
pBluescript II KS	cloning vector, Ap^r	Stratagene
pT7-7	expression vector, Ap^r	(Tabor and Richardson, 1987)
pCR-Script Amp SK(+)	cloning vector, Ap^r	Stratagene
pCR 2.1	cloning vector, Km^r	Invitrogen
pVLT35	expression vector, Sm^r	(de Lorenzo *et al*., 1993)
pAJ115	*dxnA1A2-dxnB* genes, Tc^r	(Armengaud *et al*., 1998)
pAJ114	*dbfB* gene, Tc^r	(Armengaud *et al*., 1998)
pAJ130	*redA2 -fdx1* genes, Ap^r	(Armengaud and Timmis, 1998)
pRW1	*Sphingomonas* RW1 plasmidic gene library, Ap^r, *edo2* gene	(Happe, data not published)
pRW4	*Sphingomonas* RW1 plasmidic gene library, Ap^r, *edo3-H3* genes	this work
pBl4A/B	3.1 Kb *Sac*I subcloned from pRW4, Ap^r, *edo3-H3* genes	this work
pBl5A/B	1,270 bp *Eco*RI subcloned from pRW4, Ap^r	this work
pBl6	0.8 Kb *Eco*RI-*Pst*I, subcloned from pRW4, Ap^r	this work
pBl7	1,945 bp *Eco*RI-*Sac*I subcloned from pRW4, Ap^r	this work

pCRW4	*edo3*, Ap^r	this work
pT7W4	Construct used for Edo3 overexpression, Ap^r	this work
pSK-22	*edo2-2*, Ap^r	this work
pSK-23	*edo2-3*, Ap^r	this work
pT7-22	Construct used for Edo2-2 overexpression, Ap^r	this work
pT7-23	Construct used for Edo2-3 overexpression, Ap^r	this work

2.4 Culture media and buffer solutions

2.4.1 Complex media

LB-Medium (Luria Bertani medium)	Bacto-Trypton	10g
	Yeast extract	5g
	NaCl	5g
	to 1 l	pH 7.0

Minimal medium

Components for the minimal medium:

2 x Buffer	K_2HPO_4 x12 H_2O	
(50 mM phosphate buffer pH 7.6)	KH_2PO_4	
	to 1 l with MilliQ-H_2O	
20 x Buffer	10 x concentrated 2 x buffer	
Trace elements(sterile filtrated)	HCl (25 %)	1.3 ml
	$ZnCl_2$	70 mg
	$MnCl_2$ x $4H_2O$	100 mg
	H_3BO_4	62 mg
	$CoCl_2$ x $6H_2O$	190 mg
	$CuCl_2$ x $2H_2O$	17 mg

	$NiCl_2$ x $6H_2O$	24 mg
	$NaMoO_4$ x $2H_2O$	36 mg
	to 1 l with MilliQ-H_2O	
100 x Salt-solution	$Ca(NO_3)_2$ x $4H_2O$	5 g
	$(NH_4)2SO_4$	100 g
	$MgSO_4$ x $7H_2O$	20g
	Fe-ammonium-citrate (28 % Fe)	1g
	to 900 ml with MilliQ-H_2O	
	Trace elements solution	100 ml

Assembling of the minimal medium

Liquid medium	99 % (v/v)	2 x Buffer
	1 % (v/v)	100 x Salts
	C-source in the appropriate concentration	
Solid medium	10 % (v/v)	20 x buffer
	1 % (v/v)	100 x Salts
	1.5 % (w/v) purified agar (OXOID Limited, Basingstoke, Hampshire, England)	
	C-source in the appropriate concentration in MilliQ-H_2O	

E. coli cells were cultured at 30-37 °C in liquid medium containing the appropriate selection markers on a rotary shaker operated at 160 rpm, in Erlenmeyer flasks filled up with no more than 20 % of their total volume.

Sphingomonas RW1 was cultured in liquid minimal medium with dioxin or dibenzofuran as carbon sources (0.1 % w/v) provided as crystals inside of glass tubes fixed to the cap of the erlenmeyer flasks. The two substrates diffused through the air phase to the cell suspensions.

2.4.2 Selection markers

Antibiotics:

Ampicillin (Ap)	1000 x solution	100 mg/ml in MilliQ-H_2O
	final concentration	100 µg/ml
Chloramphenicol (Cm)	1000 x solution	30 mg/ml in ethanol
	final concentration	30 µg/ml
Spectinomycin (Sp)	1000 x solution	30 mg/ml in MilliQ-H_2O
	final concentration	30 µg/ml

Xgal stock solution	20 mg/ml in dimethylformamide
	stored at -20°C
	50 µl added to each agar plate

IPTG	stock solution	10 mM
	concentration used	1 mM

2.4.3 Other solutions

50 x TAE	2M Tris + 50 mM EDTA + 57.1 ml acetic acid
K/PO_4	50 mM K_2HPO_4/KH_2PO_4, pH 7.0-9.0
Na-acetate$^-$	20 mM acetic acid + NaOH, pH 5.0
TE	10 mM Tris/HCl, 1 mM EDTA, pH 8.0
Tris/HCl	100 mM Tris + HCl, pH 7.0-9.0

2.5 Genetic methods

Standard DNA manipulations were carried out essentially according to Sambrook *et al.* (Sambrook *et al*., 1989).

2.5.1 Plasmid preparation

2.5.1.1 Preparation of big plasmids

A total of 100 ml cell suspension was centrifuged (8,000 rpm, 10 min), resuspended in GTE-buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris/HCl pH 8) containing 4 µg/ml lysozyme and incubated on ice for 10 minutes. A volume of 10 ml lysis solution (NaOH/SDS) was added to the cell suspension and the mixture incubated for further 10 minutes on ice. Cell membranes and proteins were precipitated by adding 7.5 ml 3 M sodium acetate (pH 4.8) followed by centrifugation for 15 minutes at 14,000 rpm. The nucleic acid was precipitated by adding to the clear supernatant 100 ml isopropanol (100%) followed by centrifugation (14,000, 15 min). The DNA pellet was dried, resuspended in 2 ml Tris/HCl buffer (pH 8.50 mM) LiCl (5 M), incubated for 15-30 min on ice and centrifuged at 6,000 rpm for 5 minutes. A volume of 10 ml ethanol was added to the supernatant. The mixture was incubated at -20°C for 1 hour and centrifuged (14,000, 10 min.). The DNA pellet was dried and resuspended in H_2O.

2.5.1.2 Preparation of plasmids by mean of column chromatography

In this method the plasmidic nucleic acid was prepared by mean of a column chromatography. All necessary material and buffers were provided by Qiagen GmbH (Hilden, Germany). Column sizes were chosen with respect to the DNA quantity to be isolated. Qiagen tip 20, 100 and 500 columns were used to prepare plasmid DNA of up to 20, 100, 500 µg respectively. Purification was performed as suggested from the Qiagen company.

2.5.2 Removal of traces of salts by dialysis

A volume of 20 µl DNA solution was dropped on a filter membrane (Millipore, 0.025-0.05 µm) floating on MilliQ-H_2O and dialysed for 20 minutes. Salts present in the DNA suspension diffused through the membrane to the water following a concentration

gradient. DNA to be transferred into competent recipient cells through electroporation was always dialysed previous to transformation (2.5.9).

2.5.3 Polymerase chain reaction (PCR)

The PCR reactions were performed in a total volume of 50 µl solution (10 mM Tris/HCl pH 8.50 mM KCl, 0.1 % Triton X-100, 1.25 mM $MgCl_2$, 2 % DMSO) containing 50 pmol of each primer (LTI), 50 ng of template, 200 µM of dNTPs, 2 units of thermostable polymerase Pfu (Stratagene Europe, Amsterdam) or Taq polymerase (Boehringer-Roche). After an initial denaturation step at 96 °C for 3 min, the conditions for annealing, polymerization and denaturation were 42 °C for 45 sec, 72 °C for 2 min per each 1,000 bp to be amplified and 96 °C for 1 min during 25 cycles, followed by a 10 min polymerization step at 72 °C. Reactions were performed in a Perkin Elmer (GeneAmp PCR system 2400) PCR machine not requiring addition of mineral oil at the top of each raction sample. Amplified fragments were analysed and separated on a 1.5 % agarose gel. The bands of interest were excised with a lancet from the agarose gel and the DNA purified as described in 2.5.1.

2.5.4 DNA sequencing and homology research

Nucleotide sequencing of both DNA strands was carried out using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer) with double stranded templates in the presence of 5 % DMSO. Samples containing fluorescence-labeled dideoxynucleotide terminators were processed on a 373 Stretch Applied Biosystem Automated Sequencer. Sequences were compiled by using GeneWorks software from IntelliGenetics. Homology analysis between sequences was performed using the CLUSTAL_X multiple sequence alignment program. The phylogenetic unrooted tree shown in this study was drawn by using TreeView belonging to the Phylip package program.

2.5.5 Enzymatic restriction of DNA

Standard DNA manipulations were carried out essentially according to Sambrook. (Sambrook *et al*., 1989). Restriction enzymes and reagents for genetic procedures were purchased from New England Biolabs, Boehringer Mannheim, Promega, United States Biochemicals and Sigma. In each restriction reaction an amount of 0.5-3 µg DNA dissolved in 10 to 30 µl H_2O-reaction buffer solution was digested with 0.5-2 Units restriction enzyme over 1-2 hours at the optimal reaction temperature for the enzyme. The reaction buffers (10x concentrated) were provided, together with the restriction enzymes, by the producing company. When required, BSA was added to the restriction reaction at a concentration of 100 µl/ml. The resulting DNA fragments were analysed on an agarose gel (2.5.6)

2.5.6 Gel electrophoresis

The size of DNA fragments or entire plasmids was determined by analysis on 0.8-1.5 % agarose gels. The necessary amount of agarose was dissolved in 1x TAE buffer, melted and poured into an electrophoresis chamber. A volume of 10 % v/v loading buffer (60 % glycerin, 0.3 % xylolcyalnol 0,3 % (v/v) bromophenolblue) was added to the DNA samples. Samples were loaded onto the gel using as running buffer 1x TAE. The electrophoresis was performed at 20-100 Volt/cm. The agarose gel was stained in an ethidium bromide solution (1 g/ml) for 10 minutes and analysed under UV light.

2.5.7 DNA extraction from agarose gels

After separation on agarose gels, DNA fragments of interest were cut out from the gel by mean of a lancet, weighted and treated by mean of the Qiagen II Agarose Gel Extraction Kit. All buffers were provided by Qiagen GmbH (Hilden, Germany). The extraction was performed following the protocol suggested by the provider.

2.5.8 Ligation

The reaction was carried out in a total volume of 20 µl containing 50 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM Spermidine, 1 mM ATP, 0,1 mg/ml BSA, 1

unit of T4-DNA ligase and at least 100 ng each DNA fragment to be ligated. The ligation reaction was performed at room temperature for 1 hour. Alternatively, in case of ligation of large DNA fragments, the mixture was kept over night in a PCR termocycler at a constant temperature of 16 °C. An aliquot of 1 to 5 µl of the reaction mixture was then transformed into *E. coli* cells as described in 2.5.9.

2.5.9 Transfer of DNA into recipient microorganisms and screening methods

2.5.9.1 Heat shock

A volume of 200 ml LB medium was inoculated with 200 µl of an over-night culture of *E. coli*. As soon as the culture reached an OD_{600} of 0.4-0.6, it was cooled on ice for 20 minutes. Cells were harvested (GSA rotor, 7000 rpm, 19 min, 4 °C) and all following preparation steps were performed at 4 °C and with previously cooled up material. The cell pellet was resuspended in 40 ml solution 1 (10 mM Na-acetate pH 5.6, 50 mM $MnCl_2$, 5 mM NaCl) and incubated on ice for 20 minutes. This cell suspension was pelleted and resuspended in 4 ml solution 2 (10 mM Na-acetate, 5 mM $MnCl_2$, 5 % glycerin, 70 mM $CaCl_2$). Cells were stored at –70 °C in 200 µl aliquots and slowly melted on ice before usage. For each transformation 100 µl competent cells were mixed with approximately 500 ng DNA, kept on ice for at least 30 min before being heat shocked for 45 seconds at 42 °C in a water bath. After the heat shock, 1 ml LB medium was added to the cell-DNA mixture. After incubation at 37 °C for 1 hour, cells were plated on selective solid medium.

2.5.9.2 Electroporation

Electrocompetent cells were prepared as follows. A volume of 200 ml LB medium was inoculated with 200 µl of an over-night bacterial culture. The culture was harvested (GSA rotor, 7000 rpm, 19 min, 4 °C) as soon as it reached an OD_{600} of 0.6-0.8 and washed once in 200 ml and once in 100 ml cold sterile MilliQ water. Cells were then resuspended in 200 µl cold sterile MilliQ water and stored at -70 °C until usage. For the electroporation, 200 µl cells were mixed with 20-500 ng dialysed DNA and transferred

into a precooled electroporation cuvette (0.1 cm). The electroporation was performed at 1.7 kV (17 kV/cm), 25 F and 200 Ohm by mean of a "Gene Pulser" (Bio-Rad, Hercules, USA). Cells were then resuspended in 800 µl LB, left shaking at 30 °C for 1 hour and then plated on selective solid medium.

2.6 Biochemical methods

2.6.1 Screening of *meta*-cleavage activity by compound 2,3-dihydroxybiphenyl

2,3-dihydroxybiphenyl has been shown to be a substrate for many extradiol dioxygenases, including DbfB of *Sphingomonas* sp. strain RW1. The *meta*-cleavage reaction product is yellow colored providing an easy colorimetric test for a rapid screening of bacterial colonies carrying 2,3-dihydroxybiphenyl *meta*-cleavage activity. In particular a sterile solution containing 0.1 % 2,3-dihydroxybiphenyl in 10 % acetone-water was sprayed on the bacterial colonies. Positive clones could be identified because turning to yellow.

2.6.2 Protein overexpression

Overexpression of Edo2-2, Edo2-3 and Edo3, was carried out in *E. coli* BL21(DE3)[LysS] cells respectively transformed with constructs pT7-22, pT7-23 and pT7W4, carrying genes *edo2-2*, *edo2-3* and *edo3* cloned into vector pT7-7 under the control of a T7 RNA polymerase promoter Ø 10, inducible by isopropyl-thio-ß-D-galactopyranoside (IPTG). *E. coli* cells were precultured at 30 °C in Luria-Bertani medium containing 50 mg/ml of ampicillin and 30 mg/ml of chloramphenicol, on a rotary shaker operated at 160 rpm. The same medium supplemented with 1.0 mM isopropyl-thio-ß-D-galactopyranoside (IPTG) was inoculated by 0.5-2.0 % of preculture and incubated as above until an OD_{600} of 2.0 was reached. Whole cells overproducing the extradiol dioxygenases were used for expression studies (2.7) or to prepare protein crude extracts (2.6.2). *E. coli* DH101 (pRW1) (B. Happe, not published) was also used in this study for expressing and purification of Edo2 extradiol dioxygenase. Cells were treated in the same way as the overexpressing strains. Protein crude extracts were prepared as described in 2.6.2. For overexpression of DbfB extradiol dioxygenase, *E.*

coli BL21(DE3)[LysS] pT7-5-RW (Happe *et al*., 1993) was used. Overerexpression was carried out as for the other extradiol dioxygenase proteins.

2.6.3 Preparation of cell crude extracts

Cell extracts were prepared at 4 °C. For each extract 50 ml to 3 l cells were harvested by centrifugation (8000 rpm, 10 minutes), washed once in 50 mM phosphate buffer pH 7.6, resuspended in 1 ml to 10 ml of the same buffer and disrupted in a French pressure cell (Aminco Corp.) operated at 20,000 Ib/in^2. Cell debris was removed by centrifugation at 45,000 rpm for 45 min. For O_2 free preparations, all manipulations were carried out in an anaerobic chamber (Coy, Ann Arbor, Mich.), filled with 95 % N_2 and 5 % H_2. Traces of oxygen were removed by palladium catalyst. Cells were disrupted by sonication inside of the anaerobic chamber. All buffers were boiled under a stream of N_2 and supplemented with 2 mM dithiothreitol.

2.6.4 Determination of the protein content

Protein concentrations were determined by the method of Bradford (Brandford, 1976), using bovine serum albumin (BSA) as a standard. The protein preparation was diluted with H_2O to the final volume of 800 µl and mixed with 200 µl Bradford reagent (BioRad, Richmond, USA). After 8 minutes incubation at room temperature the absorbance at λ = 595 nm was measured by mean of a spectrophotometer and using zeroing against 800 µl H_2O+ 200 µl Bradford reagent. The obtained absorbance was compared with that one of a series of standard BSA dilution solutions of known concentration and the protein content deduced.

2.6.5 Determination of oxygen uptake rates

Oxygen uptake rates were determined polarographically using an oxygen electrode Hansatech CB1D (Kings Lynn, England) in a temperated (25 °C) 1 ml reaction vessel. Calibration of the electrode was performed with oxygen saturated water (100 % or 253 µM) and after addition of sodium dithionite (oxygen depleted). Oxygen uptake rates

were determined in a total volume of 1 ml potassium phosphate buffer (pH 7.5, 50 mM). Whole cells used during these experiments were washed twice with above buffer and resuspended in the buffer to an OD_{600} of approximately 10. 20-100 µl of this suspension were added to the oxygen saturated buffer and the endogenous respiration determined. The substrate dependent oxygen uptake was measured after the addition of substrate (1 mM) as the difference of complete oxygen uptake rate and endogenous respiration.

2.6.6 Spectrophotometric analyses of enzyme kinetics

An enzymatic unit (1 Unit = 1U) represent the enzymatic activity responsible for the formation of 1 µM product/min or the transformation of µM substrate/ min at 25 °C. The specific activity, was expressed as Units/g protein. Enzymatic activities were assayed by monitoring the formation of reaction products on a UV-2100 spectrophotometer (Shimadzu) over a substrate concentration range of 10 µM to 3 mM at wavelengths indicated in table 2.3 and using the respective reported extinction coefficients. Each enzymatic test was performed in a total final volume of 1 ml in 50 mM potassium phosphate buffer (pH 7.5). The measurements were started by addition of substrate. For the kinetic measurements either crude extracts or partially purified proteins were used. The kinetics constants, K_m and V_{max}, were calculated by approximation to the Michaelis Menten equation $v=V_{max} \cdot S/(K_m+S)$ with the programm KaleidaGraph.

Table 2.3: Substrates used for the enzymatic kinetics. The extrinction coefficients of the reaction products and wavelengths used for enzyme activity determinations are indicated.

Substrate	**ε product, pH 7.6 [mM^{-1} cm^{-1}]**	**λ Product (nm)**	**Reference**
2,3-Dihydroxybiphenyl	13.2	434	(Eltis *et al*., 1993)
Catechol	36	375	(Heiss *et al*., 1995)
3-Methylcatechol	16.8	382	(Heiss *et al*., 1995)
2,2',3-Trihydroxybiphenyl	22.4	434	(Happe *et al*., 1993)
2,2',3-Trihydroxybiphenyl ether	7.8	290	This work

2.6.7 SDS polyacrylamide gel-electrophoresis (SDS-PAGE)

SDS polyacrylamide gels were used for determining the molecular weight of denaturated protein subunits and to check the efficiency of the protein purifications and were prepared as follows:

5 ml sample gel (10%)	H_2O	1.9 ml
	acrylamide/N,N-methylbisacrylamide 30 %	1.7 ml
	1.5 M Tris/HCl pH 8.8	1.3 ml
	10 % SDS	50 µl
	10 % APS	50 µl
	TEMED	2 µl
2 ml staking gel (5%)	H_2O	1.15 ml
	acrylamide/N,N-methylbisacrylamide 30 %	330 µl
	0,5 M Tris/HCl pH 6.8	500 µl
	10 % SDS	20 µl
	10 % APS	20 µl
	TEMED	2 µl

Gels used in this study were always 0.2 mm thick. The buffers used were prepared as follows:

SDS running buffer:	Glycin	192 mM
	SDS	0.1 % (w/v)
	Tris/HCl	25 mM pH 8.3
loading buffer 2x	ß-Mercaptoethanol	50 µl
	Stock sample buffer	450 µl
stock sample buffer	0.5 M Tris/HCl pH 6.8	2.4 ml
	Glycerol	2.0 ml
	10 % (w/v) SDS	4.0 ml
	0.1 % (w/v) Bromophenol blue	1 ml

The protein content of the samples to be analyzed was adjusted to 1 to 30 µg. All samples were diluted 1:1 with the double concentrated loading buffer and denaturated at 95 °C for 8 minutes. Electrophoresis was performed at 15 mA for the first 10 min and at 25 mA for the rest of the run. Gels were stained with Coomassie staining solution (Sambrook *et al*., 1989) or with Roti-Blue (Roth) over night. A broad range molecular weight standard (Bio-Rad) was loaded next to the protein samples for evaluating the molecular weight of the denaturated protein subunits.

Broad range standard proteins	Phosphorylase	97.4 kDa
	BSA	66.2 kDa
	Albumine	45.0 kDa
	Carboanhydrase	31.0 kDa
	Trypsininhibitor	21.5 kDa
	Lysozym	14.4 kDa

2.6.8 Protein purification

In this study two extradiol dioxygenases of *Sphingomonas* RW1, Edo2 (variants Edo 2-2 and Edo 2-3) and Edo3 , were partially purified. All purification steps were

performed aerobically in 1 day on a fast protein liquid chromatography (FPLC) system placed in the cold room (4 °C). All buffers were supplemented with 1 mM DTT as reducing agent.

2.6.8.1 Ammonium sulfate precipitation

A saturated $(NH_4)_2SO_4$ solution in $K/Na^-PO_4^-$ buffer (50 mM, pH 7.6) was prepared by dissolving the salt crystals under continuous stirring on a heating block at 90 °C. After cooling down to room temperature, an aliquot of the saturated supernatant was gently and dropwise added to an equal volume of crude extract in a glass beaker placed on ice. The mixture was then centrifuged at 45,000 rpm for 45 minutes at 4 °C. The protein pellet containing the precipitated *meta*-cleavage activity was then dissolved in potassium phosphate buffer 50 mM, pH 7.6 and loaded onto a Pharmacia phenyl sepharose column.

2.6.8.2 FPLC (fast protein liquid chromatography)

Edo2 and Edo3 were purified in this study by mean of hydrophobic interaction chromatography (HIC) and anion exchange chromatography. Protein elution was followed by an UV detector at 280 nm. Fractions of 0.5–4 ml were collected by mean of an automatic fraction collector. The fractions were checked for *meta*-cleavage activity using as a test substrate (2.6.1). Active fractions were either further purified or used for determination of kinetic properties. Purified proteins were stored in liquid nitrogen. Active fractions obtained after each purification step were analyzed by SDS-PAGE as described in 2.6.7.

2.6.8.3 Hydrophobic interaction chromatography (HIC)

Protein Edo3 was purified in a two step process involving an initial HIC chromatography. A HiLoad 16/10 phenyl sepharose high performance column was equilibrated with 1 M ammonium sulfate/10 % ethanol in 50 mM phosphate buffer pH 7.6. The protein was eluted with a linear 0.5 M to 0 M ammonium sulfate/10 % in 50

mM phosphate buffer pH 7.6 at a flow rate of 1 ml/min. Fractions of 1 ml were collected by an automatic sample collector connected to the FPLC.

2.6.8.4 Anion exchange chromatography

In the case of purification of Edo3, the active fractions eluted from the phenyl sepharose column were concentrated 4 times and loaded onto an anion exchange column (20 ml DEAE sepharose Fast flow, Pharmacia) previously equilibrated with Tris/HCl 10 mM pH 7.6 supplemented with 10 % ethanol. The proteins were eluted with a linear gradient 0 to 0.6 M NaCl in Tris 10 mM pH 7.6 supplemented with 10% ethanol at a flow rate of 1 ml/min. In the case of partial purification of Edo2-2 and Edo2-3 from *E. coli* BL21(DE3)[LysS] (pT7-22), and *E. coli* BL21(DE3)[LysS] (pT7-23) and of Edo2 from *E. coli* HB101 (pRW1), 1 ml of the protein crude extracts was directly loaded onto a prepacked MonoQ HR 5/5 (Pharmacia) column previously equilibrated with Tris/HCl pH 7.5 50 mM supplemented with 125 mM NaCl and 2 mM $MnCl_2$. Proteins were eluted with a linear gradient of 125–500 mM NaCl in Tris/HCl pH 7.5 50 mM supplemented with 2 mM $MnCl_2$ at a flow rate of 0.80 ml/min.

2.7 Expression studies

2.7.1 Resting cell assays

2.7.1.1 Transformation of dioxin and dibenzofuran by *E. coli* DH10B (pMDK12) and *E. coli* DH10B (pMDK19)

E. coli DH10B cells, transformed with the expression systems pMDK12 or pMDK19, were precultured at 30 °C, together with the appropriate controls, *i.e. E. coli* DH10B cells and *E. coli* DH10B containing pVLT35, in Luria-Bertani medium containing 30 µg/ml spectinomycin, on a rotary shaker operated at 160 rpm. In 500 ml flasks, 100 ml of this medium supplemented with 1.0 mM isopropyl-thio-ß-D-galactopyranoside were inoculated with 0.5-2.0 % of preculture. Cells were grown under the same conditions as the preculture, harvested as soon as an OD_{600} of 2.0 was reached and washed twice with assay buffer consisting of 10 mM glucose in 0.1x M9 mineral medium. Cells were

resuspended to an OD_{600} of 2.0-10 in prewarmed assay buffer containing 0.1-0.5 mM dibenzofuran or dioxin dissolved in DMSO (Dimethylsulfoxid) (stock solution 100 mM). For quantification of dibenzofuran or dioxin transformation, cells were incubated in aliquots of 1 ml in 10 ml reagent tubes closed with teflon coated screw caps and incubated at 30 ^{o}C on an overhead shaker. After appropriate time intervals, one whole tube was analyzed for the amount of substrate remaining. For doing this, 9 ml of methanol were added for extraction of residual dibenzofuran/dioxin from cell walls and to achieve complete dissolution. In control experiments it could be shown that by this method more than 95 % of residual substrate could be extracted. Samples were centrifuged in the test tubes at 9,000 g for 20 min and the supernatant either directly analyzed by reverse phase high-performance liquid chromatography (HPLC) (2.7.2) or stored at -20°C. For quantification of water soluble substrates or transformation products, 5 ml of cell suspension were incubated in 10 ml reagent tubes closed with teflon coated screw caps and incubated at 30 ^{o}C on an overhead shaker. At appropriate time intervals aliquots of 0.5 ml were removed, centrifuged, and the supernatant either directly analyzed by HPLC or stored at -20°C.

2.7.1.2 Transformation of dioxin and dibenzofuran by mixed cultures

E. coli BL21(DE3)(LysS) (pT7-22), *E. coli* BL21(DE3)(LysS) (pT7-23), *E. coli* BL21(DE3)(LysS) (pT7W4) and *E. coli* BL21(DE3)(LysS) (pT7-5-RW) (Happe *et al*., 1993) were cultured as previously described in 2.6.2. to achieve overexpression of Edo2-2, Edo2-3, Edo3, DbfB extradiol dioxygenases respectively. *E. coli* DH10B(pMDK12) was cultured as described in 2.6.2. Induced cells were harvested at room temperature and 6,000 rpm as OD_{600} reached 2.0 and washed twice with assay buffer consisting of 10 mM glucose in 0.1x M9 mineral medium. Resting cell assays were constituted containing a final concentration of 0.1 mM dioxin, *E. coli* DH10B (pMDK12) corresponding to an OD_{600} of 5 and *E. coli* cells overexpressing a given dioxygenase corresponding to an OD_{600} of 5. Thus the final optical density of the system was 10. Cells were incubated and samples taken as described above.

2.7.2 HPLC (reverse phase high-performance liquid chromatography) analysis

This method was adopted to identify metabolites of biotransformation reactions and to quantify substrate turnover and product formation rates. The reverse phase high-performance liquid chromatography (HPLC) was operated with a Shimadzu LC-10AD liquid chromatograph system equipped with a DGU-3A degaser and a 3PD-M10A photodiode array detector. Dibenzofuran, dibenzo-*p*-dioxin and their metabolites were separated on an analytical SC 125 x 4.6 mm Lichrospher 100 RP8 5.0 µm column. An aqueous solvent system containing 0.1 % *ortho*-phosphoric acid and 36-45 % of methanol (for the identification and quantification of 2,3-dihydroxybiphenyl, 2,2´,3-trihydroxybiphenyl, 2,2´,3-trihydroxybiphenyl ether, benzoate, salicylate, catechol and quantification of 3-chroman-4-on-2-yl-pyruvate 2-pyron-6-carboxylate and 2-hydroxymuconate) 72 % of methanol (for the quantification of dioxin and dibenzofuran) or 18 % of methanol (for the identification of 3-chroman-4-on-2-yl-pyruvate, 2-pyron-6-carboxylate and 2-hydroxymuconate) was used at a flow rate of 1 ml per min (tab. 2.4). The eluent was monitored by measuring the absorption spectrum between 200 and 400 nm. Transformation products were identified by comparison of the absorption spectra and retention volumes with authentic standards. Their concentration was analyzed based on the comparison of the peak area with that of authentic standards of known concentration.

Table 2.4: typical retention volumes of substrates and products used in this study

Compound	**72 %**	**45 %**	**36 %**	**18 %**
Dibenzo-*p*-dioxin	5.3			
Dibenzofuran	2.7			
2,3-Dihydroxybiphenyl		8.8		
2,2´,3-Trihydroxybiphenyl		4.3	9.0	
2,2´,3-Trihydroxybiphenyl ether		3.9	8.3	
Benzoate		3.6	7.6	
Salicylate		4.9	10.6	
Catechol		0.9	1.7	3.3
3-Chroman-4-on-2-yl-pyruvate		1.5	3.1	
2-Pyron-6-carboxylate			0.2	0.7

3 RESULTS

3.1 Genetic characterization of *edo2* and *edo3* genes

A *Sphingomonas* RW1 genomic library constructed in the cosmid pLAFR3 (Happe *et al*., 1993) was screened for the presence of *meta*-cleavage activity by plating it, incubating the plates overnight and spraying the *E. coli* S17-1 colonies with a solution of 0.1 % 2,3-dihydroxybiphenyl (2,3-DHB) in 10 % acetone-water (2.6.1). Several colonies exhibiting activity against 2,3-dihydroxybiphenyl were identified, the corresponding genomic inserts partial digested with *Bam*HI and further subcloned into pUC18 vector precut with the same enzyme (B. Happe, data not published). The resulting plasmids were transformed into *E. coli* DH101. In particular two constructs, pRW1 and pRW4, carrying respectively a 3.3 Kb *Bam*HI and a 10.5 Kb *Bam*HI insert were further analyzed. In this study we present the genetic and biochemical characterization of two extradiol dioxygenase encoded by two genes encoded on the 3.3 Kb *Bam*HI and a 10.5 Kb *Bam*HI inserts and subsequently named Edo2 and Edo3 extradiol dioxygenases.

3.1.1 Genetic characterization of the *edo3* gene

The 10.5 Kb *Bam*HI genomic insert carried by the plasmid pRW4 was mapped through restriction analysis as follows. The plasmid pRW4 was initially subjected to single restriction by mean of *Sac*I, *Pst*I, *Eco*RI, *Sma*I, *Hind*III, *Sal*I, *Sph*I, *Xba*I restriction enzymes, respectively. The reaction samples were analyzed on a 0.8 % agarose gel. No *Hind*III, *Sph*I and *Xba*I recognition sequences and multiple *Sal*I sites were found in the insert. As *Sac*I, *Pst*I, *Eco*RI and *Sma*I were shown to cut the insert at reasonable 2-4 times, those enzymes were used in all possible combinations to perform double restrictions of pRW4. By comparing the length of the resulting DNA fragments the restriction map presented in fig. 3.1 could be deduced. Several subclones of the 10.5 Kb *Bam*HI fragment were prepared and screened for expressing an active 2,3-dihydroxybiphenyl 2,3-dioxygenase. By restriction with *Sac*I, five fragments have originated, three of which carrying only insert and no vector sequence and were

respectively 3.4 Kb, 3.1 Kb and 0.4 Kb in lenght. The 3.4 Kb and 3.1 Kb fragments were purified (2.5.7) and ligated to vector pBluescript II KS (Amp^R) previously subjected to restriction with *Sac*I. *E. coli* DH5α transformants were screened for *meta*-cleavage activity by spraying the colonies with 2,3-DHB. Bacterial colonies turning to yellow after spraying with 2,3-DHB and therefore expressing *meta*-cleavage activity, were shown to carry the 3.1 Kb *Sac*I DNA insert in pBluescript II KS, furtheron named pBl4A. Further subclones of pRW4 were constructed by restriction with *Eco*RI, *Pst*I and *Sac*I and ligation of the respective purified 1270 bp *Eco*RI, 1945 *Sac*I-*Eco*RI and 800 bp *Eco*RI-*Pst*I fragments to pBluescript II KS vector precut with the appropriate enzymes. However, none of the resulting plasmids, pBL5, pBl7, pBl6, in neither orientation of insertion into the cloning vector, expressed activity against 2,3-DHB. The 10.5 Kb *Bam*HI genomic insert was partially sequenced. In specific, 3215 base pairs delimited by *Sac*I and *Eco*RI restriction sites, as evidenced in fig. 3.1B, have been sequenced. To obtain the sequence, pBl4A, pBl5A, pBl6 and pBl7 constructs were used as DNA template and the PCR primers listed in tab. 3.1 for the sequencing reaction. A 800 bp ORF was found (fig. 3.2) in the resulting sequence which codes for a protein with homology with known extradiol dioxygenases and precisely sharing 38.6 % identity in the amino acid sequence with the biphenyl-2,3-diol 1,2-dioxygenase from *P. pseudoalcaligenes* KF 707 (BPHC_PSEPS)(Furukawa *et al*., 1987) and 38.3 % identity with the biphenyl-2,3-diol 1,2-dioxygenase of *Pseudomonas* strain LB400 (BPHC_BURCE) (Eltis *et al*., 1993). The gene was therefore named *edo3* extradiol dioxygenase. Immediately preceding the *edo3* gene an 850 bp ORF was found encoding for a hydrolase, H3, sharing 34% identity in the amino acid sequence with the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase of *Pseudomonas* LB400 (BPHD_BURCE) (Hofer *et al*., 1993)(fig. 3.1B). The deduced *H3* stop codon overlaps the *edo3* gene ATG starting codon (fig. 3.2). A ribosome binding sequence can be identified 6 nt upstream of the *H3* starting codon. In case of the *edo3* gene no evident ribosome-binding site could be found, however an A-G rich region is present between position –10 and –15 in respect of the *edo3* ATG starting codon. Possibly, in RW1 the two genes are cotranscribed. Flanking the *edo3* and *H3* genes, there are DNA regions sharing homology with known trascriptional regulators such as BM3R1 from *Bacillus Megaterium* (Shaw and Fulco, 1992) or E2 in *Papilloma Virus* (Fuchs *et al*., 1986).

Table 3.1: List of primers used for the sequence analysis as indicated in fig. 3.1B.

Name of the primers	**Primer sequence (5'-3')**
M13-20 Primer	GTAAAACGACGGCCAGT
reverse Primer	AACAGCTATGACCATG
M1	CGGTTGGTGGAGTGGGTG
M2	CACCCACTCCACCAACCG
M3	GACCCCGGGTCCGATCGC
M4	GCGATCGGACCCGGGGTC
M5	CTCTACGGCCTCCCCTGG
M6	CCAGGGGAGGCCGTAGAG
M7	GCGGATGCGGAACGGATG
M8	CATCCGTTCCGCATCCGC
M9	CTCGGCCGCCACTGCAAC
M10	GTTGCAGTGGCGGCCGAG
M11	CCCGCCTTCTCCGCGATG
M12	CATCGCGGAGAAGGCGGG
M13	CCTGCTGTTCGACGTGGC
M14	GCCACGTCGAACAGCAGG
M15	CCATCAGCACCAGTCCCTCG
M16	CAACGGCTCAGGCATTGCG
M17	GTCGGCAACTCGCTCGGC
M18	GTCGGCAACTCGCTCGGC
M20	CATCTCCAGCATTTCCTCAC
M21	CGGTCAGGGGCCGTCGGC

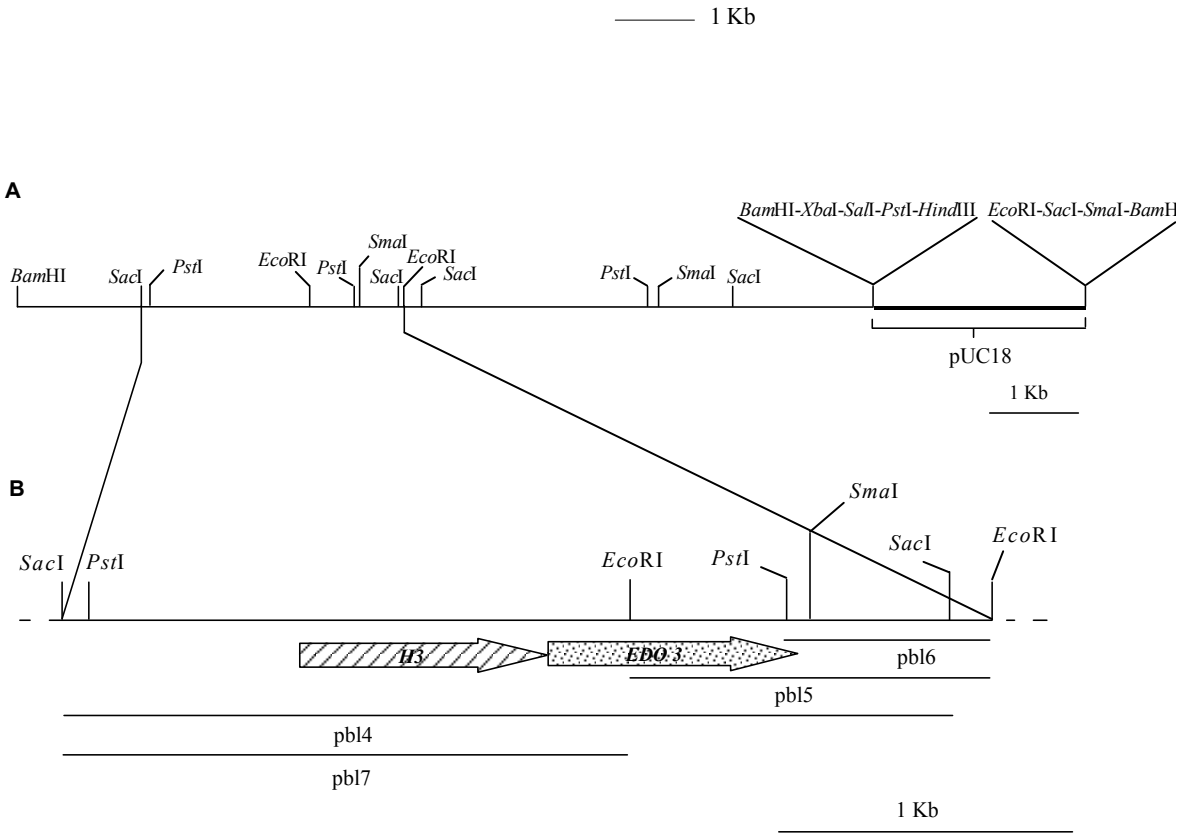
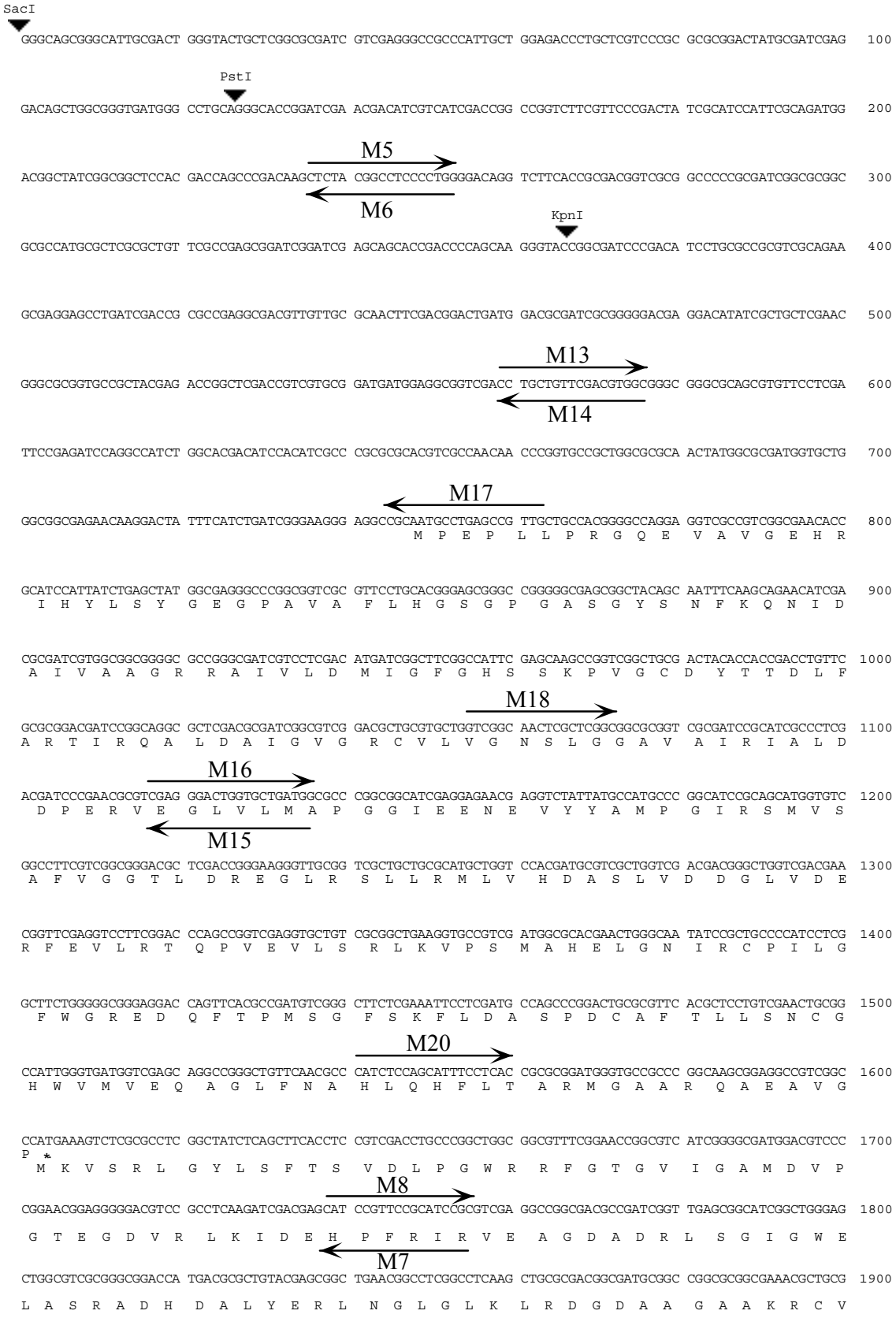


Fig. 3.1: A:restriction map of the 10,5 Kb *Bam*HI DNA insert carried by pRW4 plasmid; B: detail of the sequenced region. By sequencing the two ORFs encoding for H3 and Edo3 proteins have been localized in the position shown on the map. The localization of subclones pBL4, pBL5, pBL6, pBL7 is also shown.


SacI
PstI
M5
M6
KpnI
M13
M14
M17
M18
M16
M15
M20
M8
M7

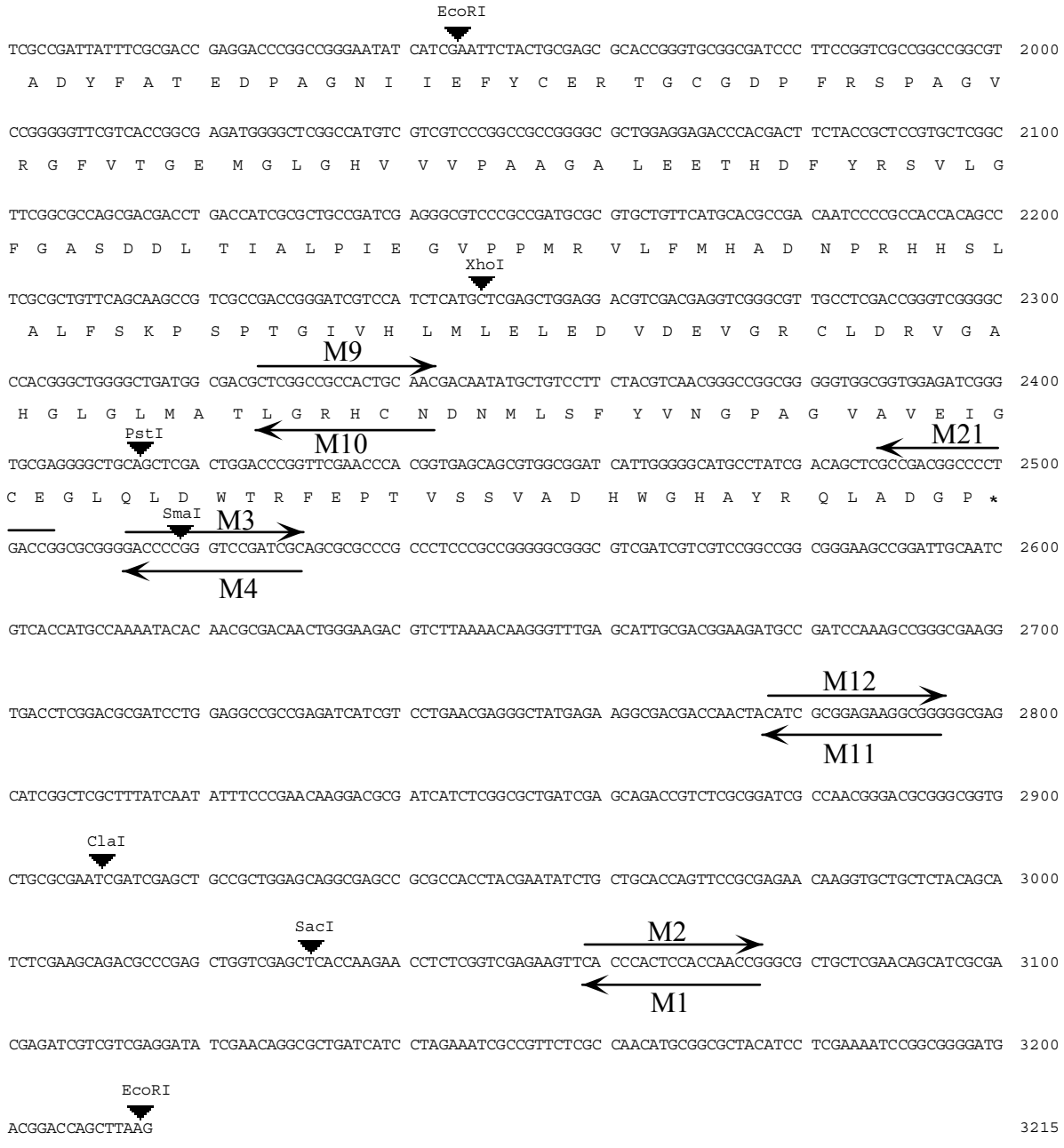


Fig. 3.2: Complete sequence of the 3215 bp *Sac*I-*Eco*R1 fragment indicated in fig. 3.1B. The primers used for the sequencing reactions and their specific orientation are indicated by arrows. The DNA regions encoding *H3* and *edo3* genes have been translated into amino acid sequences. The gene stop codons are indicated with a "*".

3.1.2 Genetic characterization of the *edo2* gene

The 3.3 Kb *Bam*HI genomic insert carried by the plasmid pRW1 (3.1) has been mapped by restriction analysis (B. Happe, data not published) by mean of the restriction enzymes *Eco*RI, *Pst*I, *Bam*HI and *Sal*I (fig. 3.3). By sequence analysis a 892 nt ORF has been found encoding for a protein sharing homology with known extradiol dioxygenases and precisely sharing 42 % identity in the amino acid sequence with the biphenyl-2,3-diol 1,2-dioxygenase from *Rhodococcus globerulus* P6 (Asturias *et al*., 1994) and the biphenyl-2,3-diol 1,2-dioxygenase from *Pseudomonas pseudoalcaligenes* (Furukawa *et al*., 1987) and thus named Edo2 extradiol dioxygenase (fig.3.4). Interestingly, the gene sequence presents three putative ATG starting codons indicated in fig. 3.4 as ATG1, ATG2 and ATG3. ATG2 directly follows ATG1, while ATG3 is located 18 bp downstream of ATG2. No clear ribosome-binding site could be identified at the appropriate position upstream of ATG3 and ATG1. However a region rich in adenosines and guanosines is present 10-5 bp upstream of ATG2, possibly indicating ATG2 to be the proper start codon in RW1. In this work we addressed the question which of the three putative starting codons is actually used during *edo2* gene transcription in *Sphingomonas* RW1. Since it is unlikely for a protein to start with two methionines, we decided to focus on ATG2 and ATG3 as putative start codons. The possible protein variants with ATG2 and 3 as start codons and respective DNA ORFs of 889 nt and 868 nt in length, were furtheron named Edo2-2 and Edo2-3.

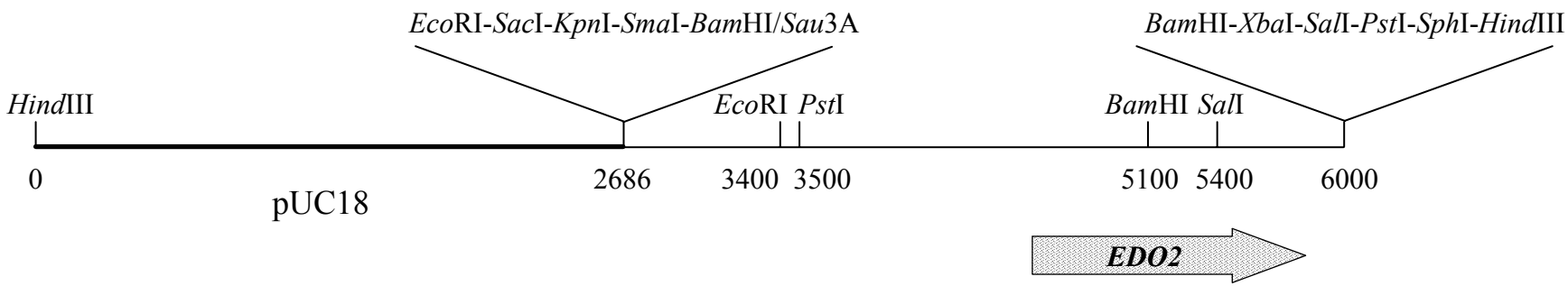


Fig 3.3: pRW1 restriction map. By sequencing analysis the *edo2* gene could be positioned as indicated in the figure.

```
ACGGGGCTAT TATGACGACG TCGTCACGGT CGATGGTGGG ACGGCCCTCT TCGCAAGCAA     60

                                                              ATG1
GACGGTCTAC ATCGACGGAA CGCCCACCCG CTATCTCGCC TATCCGCTTT GAGAAGAATG    120
                                                               M
ATG2                          ATG3
ATGACAGACC AACAGTCGAA TATGCCGTCG CCGATCGTCG CCCTGGGCTA TCTGGGTTTC    180
M  T  D  Q   Q  S  N   M  P  S   P  I  V  A   L  G  Y   L  G  F

TATGCGCAGG ATCTCGCCGC GTGGGAGGAA TGGGCGACCC AGATTTTCGG GTTCGCGAAG    240
Y  A  Q  D   L  A  A   W  E  E   W  A  T  Q   I  F  G   F  A  K

GTTCGCGCGC CGGAAGGCAT TTCCAGCGAT CATCTCTACC TGCGGATCGA CGAGCGCCAA    300
V  R  A  P   E  G  I   S  S  D   H  L  Y  L   R  I  D   E  R  Q

TGGCGCTTCG CGGTCGAGCC CGGTACGGAA GGCCGCGTCG CTTTCATCGG CTGGGAGGCC    360
W  R  F  A   V  E  P   G  T  E   G  R  V  A   F  I  G   W  E  A

GCCGACGCCA ACGCGTTGGA GGAGGTCCAT CGGCGGCTCA CCGCCCAGGG GATCGAGGTC    420
A  D  A  N   A  L  E   E  V  H   R  R  L  T   A  Q  G   I  E  V

GTCAGGGATG CCGAACTGGC GCGAAAGCGC TGCGTGCAGG ACCTGATCCG CTGCGAGGAT    480
V  R  D  A   E  L  A   R  K  R   C  V  Q  D   L  I  R   C  E  D

CCCGACGGCT TCCGGCTGGA GTTCTTCCAC GGCCATCTCG TTTCCCGCGA GCCGTTCGTC    540
P  D  G  F   R  L  E   F  F  H   G  H  L  V   S  R  E   P  F  V

TCTCCGCGCG GCATCGGCTT CGTCACCGGC GACATGGGGC TCGGCCACAT CCTCATCACG    600
S  P  R  G   I  G  F   V  T  G   D  M  G  L   G  H  I   L  I  T

GTGAGCGACA TCGAAAAGTC GAAGGCCTTC TACCTGGACC TGCTCGGCTT CAGGATGAGC    660
V  S  D  I   E  K  S   K  A  F   Y  L  D  L   L  G  F   R  M  S

GATTACATCG TCTTCGGCGG GAACAAGGTG CATTTCACCC ACATCAACCC GCGTCACCAC    720
D  Y  I  V   F  G  G   N  K  V   H  F  T  H   I  N  P   R  H  H

AGCCTGGCCT TCGTCCAGAC CAGCGATCGC ATCGCGCGGC TGGGCCACTT CATGGTGGAG    780
S  L  A  F   V  Q  T   S  D  R   I  A  R  L   G  H  F   M  V  E

GCCGACGATG TCGACGCCGT CGGCTTCGCG CTCGACCGTC TTCATGCCAG CACATGGCAG    840
A  D  D  V   D  A  V   G  F  A   L  D  R  L   H  A  S   T  W  Q

CTGAAGGAGA CCCTCGGGCG CCACACCAAT GATCGCATGC TCTCCTTCTA CTGCGAAAAT    900
L  K  E  T   L  G  R   H  T  N   D  R  M  L   S  F  Y   C  E  N

CCTTCGGGGT CGCAGACGGA GTTCGGTTGG GGCGGGCGCA AGATCGCCCA CCCCGGCTGG    960
P  S  G  S   Q  T  E   F  G  W   G  G  R  K   I  A  H   P  G  W

CTGGTGGAAA CCTATGATGC GACCGCATTC TGGGGACACA AGGTTCCGGG CACCGAATAT   1020
L  V  E  T   Y  D  A   T  A  F   W  G  H  K   V  P  G   T  E  Y

TCGGACAGGG GTCCTCAGTC TCCACGCGAA GGAATTTGAA AATGGCGGAA AAGATTCGGG   1080
S  D  R  G   P  Q  S   P  R  E   G  I  *

TGTGCGCGGA GGGCGAGATC GAGGACGGCG GCTTCAAGGG TCATCGCCCT CAGGGAACCG   1140
```

Fig. 3.4: Edo2 gene and protein sequence. The three putative ATG codons and the SD sequence are indicated.

3.1.3 Protein alignment of Edo2 and Edo3 with known extradiol dioxygenases

The amino acid sequences of Edo3, Edo2-2 and Edo2-3 were deduced directly from the DNA sequence determined and compiled by mean of the GeneWork program. The Edo2-2 protein sequence differs from that one of Edo2-3 by 7 additional amino acids at the N-terminus. The protein sequences of Edo3, Edo2-2 and Edo2-3 were aligned with those of other known extradiol dioxygenases available in public databases by mean of the CLUSTAL_X program (Thompson *et al*., 1996) (fig 3.5). The 7 additional amino acids present at the N-terminal domain of Edo2-2 extradiol dioxygenase are pushed out of the protein alignment as almost an exception in respect to all the other analyzed sequences. The protein alignment created by CLUSTALX was then used to generate the phylogenetic tree shown in fig. 3.6 by the program PRODIST of the PHYLIP (Felsenstein, 1985) package. Previously (Harayama *et al*., 1992) extradiol dioxygenases have been classified into two groups in respect of the substrate preference for either bicyclic or monocyclic substrates. It is now becoming clear that such division is only an approximation of the real more complex situation, as evidenced by the increasing number of three-dimensional studies conducted on a large number of extradiol dioxygenases, which revealed the presence of several subclasses of enzymes (Eltis and Bolin, 1996). However, only for a limited number of extradiol dioxygenases the substrate spectrum has been definitely characterized, and thus it is not clear whether the different subfamilies described by Eltis differ also in their respective catalytic properties. We can however observe, without any strong classification purpose, that Edo2-2, Edo 2-3 and Edo3 clearly cluster with several extradiol dioxygenase which are known to prefer bicyclic aromatics as substrates (Eltis and Bolin, 1996) such as: the 2,3-dihydroxybiphenyl 1,2-dioxygenases from *P. pseudoalcaligenes* KF 707, the 2,3-dihydroxybiphenyl 1,2-dioxygenases from *P. cepacia* LB400, 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Pseudomonas sp.* strain KKS102, the 2,3-dihydroxybiphenyl 1,2-dioxygenases from *R. globerulus* P6.

```
DMPB_PSEPU ---------- MKKGVMRPGH VQLRV----- -------LN- LESALAHYRD
XYE2_PSEPU ---------- MKKGVMRPGH VQLRV----- -------LN- LEAALTHYRD
XYE1_PSEPU ---------- MNKGVMRPGH VQLRV----- -------LD- MSKALEHYVE
XYLE_PSEAE ---------- MNKGIMRPGH VQLRV----- -------LD- MSKALEHYVE
NAHH_PSEPU ---------- MNKGVMRPGH VQLRV----- -------LD- MGKALEHYVE
BHC1_RHOGO ---------- --MSVQRLGY LGIEV----- -------SD- VDAWRTYATM
TODE_PSEPU ---------- --MSIQRLGY LGFEV----- -------AD- VRSWRTFATT
BPHC_BURCE ---------- --MSIRSLGY MGFAV----- -------SD- VAAWRSFLTQ
BPHC_PSEPS ---------- ---SIRSLGY MGFAV----- -------SD- VAAWRSFLTQ
BPHC_PSES1 ---------- ---SIERLGY LGFAV----- -------KD- VPAWDHFLTK
EDO2-3     ---------- MPSPIVALGY LGFYA----- -------QD- LAAWEEWATQ
EDO2-2     ---MTDQQSN MPSPIVALGY LGFYA----- -------QD- LAAWEEWATQ
BPHC_PSEPA ---------- -MVAVTELGY LGLTV----- -------TN- LDAWRSYAAE
BPH_RHA1   ---------- -MAKVTELGY LGLSV----- -------SN- LDAWRDYAAG
NAHC_PSEPU --------MS KQAAVIELGY MGISV----- -------KD- PDAWKSFAMN
BHC2_RHOGO ---------- -MTATPKFAH VVLQT----- -------SR- FEAMRDWYCT
BHC3_RHOGO ---------- -MTVTPRLAH FVLQT----- -------NQ- LPAMTQWYID
DBF_DPO360 ---------- -MSKVKELAY VGYEV----- -------SD- LAAWEHFGVD
BPH_P20    ---------- -MQSVSTLGY MVIGV----- -------SD- LAAWESFAVN
EDO3       ---------- --MKVSRLGY LSFTS----- -------VD- LPGWRRFGTG
dbfB_new   ---------- --MSVKQLGY LIFECR---- -------ADV LEQMVVVYQD
CDO_CTM    ---------- MSNITSDSNI VEVSVPRVHN LHHVELLTPK PNESLDFFTR
PHEB_BACST ---MSKNFQE PIFDVAQLAH VELLS----- -------PK- LEESIVFFTK
DPA_ARTGLO MTNFVPTPSV PAPDIVRCAY MEIVV----- -------TD- LAKSREFYVD
BPH_BN6    ----MSQTET SPIRVEKIAH IVLFV----- -------KD- PELSAQWYSD
```

Fig 3.5: Edo2-2, Edo2-3 and Edo3 protein sequences were aligned with those of known extradiol dioxygenases (see also fig. 3.6 and the respective legend). In the figure only the N-terminal segment of the alignment is shown.

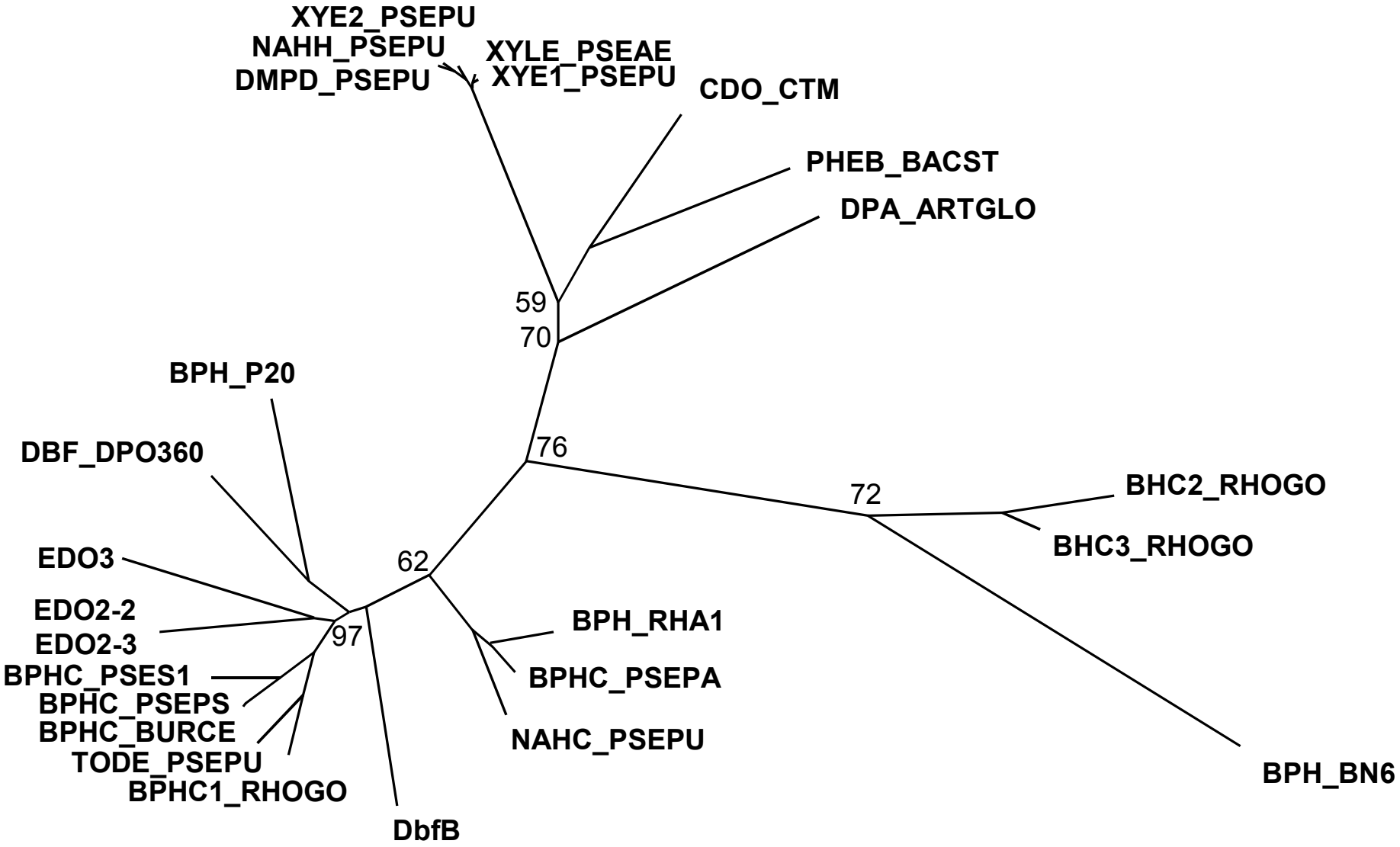

XYE2_PSEPU
NAHH_PSEPU
DMPD_PSEPU
XYLE_PSEAE
XYE1_PSEPU
CDO_CTM
PHEB_BACST
DPA_ARTGLO
59
70
BPH_P20
DBF_DPO360
76
72
BHC2_RHOGO
BHC3_RHOGO
EDO3
62
EDO2-2
EDO2-3
97
BPH_RHA1
BPHC_PSES1
BPHC_PSEPS
BPHC_BURCE
TODE_PSEPU
BPHC1_RHOGO
BPHC_PSEPA
NAHC_PSEPU
BPH_BN6
DbfB

Fig. 3.6: Phylogenetic tree obtained from the alignment of Edo2-2, Edo2-3 and Edo3 with related extradiol dioxygenases. The protein sequences were first aligned using the CLUSTELX program. The phylogenetic analysis was performed using the neighbor-joining algorithm of PHYLIP . Distance matrices were generated with the PRODIST program. The phylogenetic unrooted tree was drawn with DRAWTREE of the PHYLIP. The branch length correlate with the percentage divergence of the protein sequences, The numbers indicate the confidence (percent) estimated by bootstrap analysis with SEQBOOT program on 100 replications. Proteins are labeled by abbreviations. Their accession codes in the SwissProt or GeneBank databases and their origins are X60740 for catechol 2,3-dioxygenase from *Pseudomonas aeruginosa* JI104 (XYLE_PSEAE), V01161 for catechol 2,3-dioxygenase from *P. putida* MT-2 (XYE1_PSEPU), M17159 for catechol 2,3-dioxygenase from *P. putida* PpG7 (NAHH_PSEPU), U33263 for catechol 2,3-dioxygenase from *Pseudomona*s CF600 (DMPB_PSEPU), M65205 for catechol 2,3-dioxygenase from *P. putida* (XYE2_PSEPU), X75634 for 2,3-dihydroxybiphenyl dioxygenase II from *R. globerulus* P6(BHC2_RHOGO), X75635 for 2,3-dihydroxybiphenyl dioxygenase III from *R. globerulus* P6 (BHC3_RHOGO), X69504 for catechol 2,3-dioxygenase from *Rhodococcus rhodochrous* CTM (CDO_CTM), X67860 for catechol 2,3-dioxygenase from *Bacillus stearothermophilus* FDTP-3 (PHEB_BACST), D76438 for 2,3-dihydroxybiphenyl dioxygenase from *Rhodococcus* sp. strain RHA1 (BPH_RHA1), M20640 for 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas paucimobilis* Q1 (BPHC_PSEPA), J04994 for 1,2-dihydroxynaphthalene dioxygenase from *P. putida* PpG7 (NAHC_PSEPU), X75633 for 2,3-dihydroxybiphenyl 1,2-dioxygenases from *R. globerulus* P6 (BPHC1_RHOGO), J04996 for 3-methylcatechol 2,3-dioxygenase from *P putida* F1 (TODE_PSEPU), M15333 for 2,3-dihydroxybiphenyl 1,2-dioxygenases from *P. pseudoalcaligenes* KF 707 (BPHC_PSEPS), X66122 for 2,3-dihydroxybiphenyl 1,2-dioxygenases from *P. cepacia* LB400 (BPHC_BURCE), M26433 for 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas* sp. strain KKS102 (BPHC_PSES1).U22355 for 2,3-dihydroxybiphenyl dioxygenase from naphthalenesulfonate -degrading bacterium strain BN6 (BPH_BN6), JC4922 for 2,3-dihydroxybiphenyl dioxygenase from *P.* sp. strain P20 (BPH_P20), U57649 for dibenzofuran extradiol dioxygenase from *Terrabacter* sp. strain DPO360 (DBF_DPO360), U19817 for 3,4-dihydroxyphenylacetate 2,3-dioxygenase from *Arthrobacter globiformis* (DPA_ARTGLO).

3.2 Biochemical characterization of Edo2-2, Edo2-3 and Edo3 extradiol dioxygenases

3.2.1 Overexpression of Edo3

Gene *edo3* was amplified from pRW4 by mean of primers M13 (CATATGAAAGTCTCGCGCCTCGG, forward) and M14 (TTCGAACCCGGGGTCCCCGCGC, reverse) introducing respectively an *Nde*I site overlapping the *edo3* ATG starting codon and a *Hind*III site 19 nt downstream of the stop codon. For the amplification conditions described in 2.5.3 have been used. The resulting 828 bp blunt end DNA fragment was ligated into vector pCR-Script Amp SK(+) precut with *Srf*I giving the construct pCRW4. The amplified gene was sequenced on both strands in order to check the integrity of the genetic information. The gene *edo3* was then excised from pCRW4 by mean of *Nde*I and *Hind*III restriction enzymes and inserted into the expression vector pT7-7, previously double restricted with *Nde*I and *Hind*III, originating plasmid pT7W4. *E. coli* DH5α was used for propagating both pT7W4 and pCRW4 constructs. Overexpression of Edo3 extradiol dioxygenase was carried out in *E. coli* BL21(DE3)LysS as previously described (2.6.2).

3.2.2 Aerobic purification of Edo3

E. coli cells overexpressing Edo3 (2.6.2) were harvested and washed twice in potassium phosphate buffer pH 7.6. A quantity of 10ml crude extract was prepared in the same buffer from 3 liters cell pellet. The dioxygenase activity was precipitated from crude extract by addition of saturated ammonium sulfate solution as described in 2.6.8.1. The resulting protein pellet was resuspended in phosphate buffer pH 7,6 and loaded on a phenyl sepharose Hiload 16/10 Pharmacia column (2.6.8.2). The protein was eluted with a gradient of 0.5 M to 0 M ammonium sulfate and precisely at a 100 mM concentration of ammonium sulfate. Samples showing activity against 2,3-dihydroxybiphenyl were pooled, concentrated 4 times and loaded on a DEAE sepharose anion exchange column. The protein was eluted using a gradient from 0 mM to 600 mM NaCl and precisely at 200 mM concentration NaCl. Samples from each purification step, and showing significant activity against 2,3-dihydroxybiphenyl, were analyzed by SDS-PAGE (fig.3.7). 9% of the initial total activity could be recovered after

hydrophobic interaction chromatography, and a remaining 1.3 % activity was recovered after additional anion exchange chromatography (tab.3.2) showing protein Edo3 to be very unstable as previously reported for other extradiol dioxygenases such as DbfB (Happe *et al*., 1993).

Tab. 3.2: Partial purification of Edo 3 from *E. coli* BL21(DE3)[LysS] (pT7-W4). 10 ml of cell extract with the indicated activity when measured with 100 µM of 2,3-dihydroxybiphenyl was applied onto a phenyl sepharose Hiload 16/10 Pharmacia. Active recovered fractions were pooled and loaded onto an anion exchange column (DEAE sepharose Fast flow), and eluted as described in 2.6.8.4.

	Total protein content (mg)	**Total activity (U[a])**	**Specific activity (U/g)**	**Recovery (%)**
Crude extract	355	1030	2900	100
Phenyl sepharose	55	92	1670	9
DEAE Sepharose	8.5	13.5	1590	1.3

[a] One unit is defined as the amount of protein that converts 1 µmol of 2,3-DHB per min.

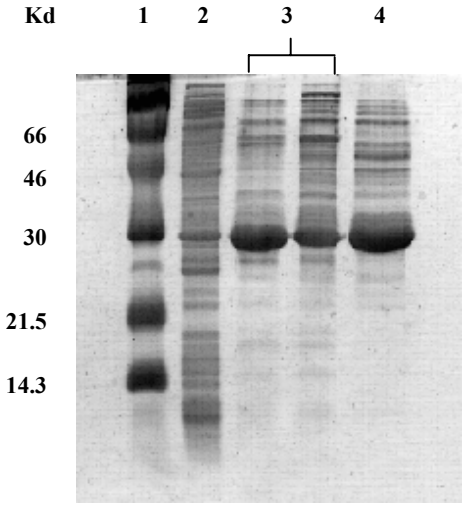


Fig 3.7: SDS-PAGE monitoring the purification of Edo3 extradiol dioxygenase. Each sample loaded on the gel corresponds to a purification step: 1, molecular weight standard (765 multicolor by Amersham); 2, *E. coli* BL21(DE3)(pLysS)(pT7W4) crude extract; 3, phenyl sepharose eluate before and after being concentrated 4 times; 4, DEAE Sepharose eluate. In all lanes an amount corresponding to 30 µg total protein content has been applied.

3.2.3 Hyperexpression of Edo 2-2 and Edo2-3

Plasmid pRW1 was used as a template for the amplification of *edo2-2* and *edo2-3* ORFs by mean of primers C230-2 (CATATG$_2$ACAGACCAACAGTCGAATATG$_3$) and C230-3 (GATG$_2$ACAGACCAACAGTCGCATATG$_3$), Primers C230-2 and C230-3 introduced an *Nde*I site overlapping ATG2 on C230-2 and ATG3 on C230-3 respectively making then possible to clone the resulting PCR products carrying *edo2-2* and *edo2-3* respectively into the expression vector pT7-7, precut *Nde*I. By looking at the primer design (where the *Nde*I site has been underlined and ATG2 and 3 evidenced) it is possible to notice that, although both ATG2 and ATG3 are present on both primers C230-2 and C230-3, only ATG3 will eventually be present on *edo2-3* cloned into pT7-7 vector, since ATG2 will fall outside of the fragment amplified by mean of C230-3 and following NdeI restriction. On the other side both ATG2 and ATG3 will be present on *edo2-2* once cloned into pT7-7 since ATG3 falls inside *edo2* amplified by mean of C230-2 primer and resulting from *Nde*I restriction. Gene *edo2-2* was therefore

eventually carrying both ATG2 and ATG3 while *edo2-3* was carrying only ATG3 but not ATG2. The PCR reactions were carried out as described in 2.5.3. The resulting 1018 bp and 1016 bp blunt-end DNA fragments were inserted into pCR-Script Amp SK(+) precut with *Srf*I, originating the recombinant plasmids pSK-22 and pSK-23, carrying respectively in the first case both ATG2 and ATG3 and in the second case only ATG3. The inserts were sequenced on both strands in order to check the integrity of the nucleic acid information. Plasmids pSK-22 and pSK-23 were digested with *Nde*I. The resulting 1018 bp and 997 bp DNA fragments, carrying the ORFs of the two genes beginning exactly with ATG2 and ATG3 respectively, were ligated to plasmid pT7-7 previously subjected to restriction with *Nde*I, originating the two recombinant plasmids pT7-22 and pT7-23 respectively. *E. coli* BL21(DE3)[LysS] cells were transformed with pT7-22 and pT7-23 constructs. Overexpression of Edo2-2 and Edo2-3 extradiol dioxygenases was carried out as previously described (2.6.2).

3.2.4 Investigation of the Edo2 protein native size

In order to analyze which of the two Edo2 protein versions, i.e Edo2-2 or Edo2-3 would be actually synthesized in *Sphingomonas* RW1, both Edo2-2 and Edo2-3 extradiol dioxygenases were partially purified from *E. coli* BL21(DE3)[LysS] (pT7-22) and *E. coli* BL21(DE3)[LysS] (pT7-23) cells, respectively and compared to Edo2 purified from *E. coli* DH101 (pRW1). In the case of *E. coli* DH101 (pRW1), 2000 bp genomic information are present upstream of the *edo2* gene, including therefore all putative *edo2* ATG starting codons and the wild type ribosome binding site which would be used during the gene expression in *Sphingomonas* RW1. Transcription start at ATG 2 would result in a molecular mass of the enzyme product of 1 kDa higher compared to a transcription start at ATG3. Such a difference should be visible after SDS-PAGE. A volume of 3 ml crude extract in potassium phosphate buffer (50 mM pH 7.5) was aerobically prepared from 200 ml induced cells of each *E. coli* strain (2.6.3). 1 ml of these protein preparations, containing 15-25 mg of protein, was loaded separately onto a MonoQ HR 5/5 (Pharmacia) anion exchange column (2.6.8.4). Proteins were eluted with a gradient from 125 mM to 500 mM NaCl. Fractions of 0.5 ml were collected and those containing the highest activity against 2,3-dihydroxybiphenyl, eluting at approximately 250 mM NaCl, were analyzed by SDS-PAGE. As shown in fig. 3.8, strong protein bands at molecular weights of about 34-35 kDa appeared in all lanes

where fractions containing activity against 2,3-dihydroxybiphenyl have been subjected to electrophoretic analysis. The molecular weight of the extradiol dioxygenase expressed from pRW1 (lane 6) appears to be identical in size to that expressed from pT7-22 (lane 5) but significantly larger than that expressed from pT7-23 (lane 4). Analysis of mixtures of fractions containing extradiol dioxygenase expressed from pRW1 and either extradiol dioxygenase expressed from pT7-23 (lane 2) or from pT7-22 (lane 3) clearly evidenced that the enzyme expressed from pRW1 is different in size from that expressed from pT7-23 (two bands of proteins with molecular weights of about 34-35 kDa) but obviously identical in size to that expressed from pT7-22 (one band of proteins with molecular weights of about 35 KDa). This indicates Edo2-2 to be the most likely form of Edo2, produced in *Sphingomonas* RW1.

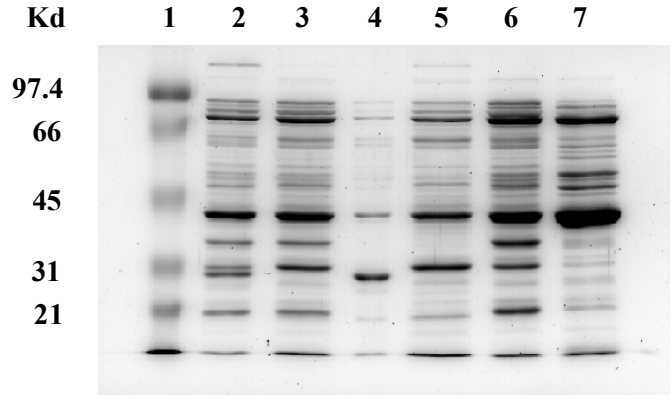


Fig. 3.8: SDS-PAGE analysis of partially purified Edo2 extradiol dioxygenases expressed from different vectors. Cell extracts from *E. coli* HB101 (pRW1), *E. coli* (DE3)(LysS)(pT7-22) and *E. coli* BL21(DE3)(LysS)(pT7-23) were subjected to ion exchange chromatography on a MonoQ HR 5/5 (Pharmacia) column and active fractions analyzed for the size of the expressed extradiol dioxygenase. The samples loaded onto the gel correspond to: 1, molecular weight marker low range (Bio-Rad); 2, Edo2 containing fraction from *E. coli* HB101 (pRW1) mixed with Edo2-3 containing fraction from *E. coli* BL21(DE3)LysS (pT7-23); 3, Edo2 containing fraction from *E. coli* HB101 (pRW1) mixed with Edo2-2 containing fraction from *E. coli* BL21(DE3)LysS (pT7-22); 4, Edo2-3 containing fraction from *E. coli* BL21(DE3)LysS (pT7-23), 5, Edo2-2 containing fraction from *E. coli* BL21(DE3)LysS (pT7-22); 6, Edo2 containing fraction from *E. coli* HB101 (pRW1); 7, *E. coli* BL21(DE3)(LysS) protein fraction eluted at the same NaCl concentration at which the extradiol dioxygenases were eluted.

3.2.5 Kinetic studies

The activity of the different extradiol dioxygenases, in freshly prepared *E. coli* BL21(DE3)LysS cell free extracts (2.6.3), overexpressing the respective enzymes was always 9.000 +/- 2000 U/g protein for DbfB, 7500 +/- 1500 U/g protein for Edo 2-3, and significantly lower in the case of Edo 2-2 (2700 +/- 700 U/g protein) or Edo 3 (2100 +/- 800 U/g protein) when measured with 100 µM 2,3-dihydroxybiphenyl as substrate. Activity in a cell free extract of Edo2 expressed by *E. coli* HB101 (pRW1) and measured under the same conditions showed only activities of approximately 100 U/g protein. It can thus be concluded that the proteins were obviously overexpressed under the control of the T7 promoter, but that either overexpression was less effective for Edo2-2 and Edo3 or that the proteins were less effective compared to DbfB and Edo2-3.

Table 3.3: Partial purification of Edo2-2 from *E. coli* BL21(DE3)[LysS] (pT7-22), Edo2-3 from *E. coli* BL21(DE3)[LysS] (pT7-23) and Edo2 from *E. coli* HB101 (pRW1) by anion exchange chromatography. 1 ml of cell extract showing the indicated activities, when measured with 100 µM of 2,3-dihydroxybiphenyl, was applied onto of MonoQ anion exchange column and eluted as described in 2.6.8.4.

	Edo 2-2	Edo 2-3	Edo 2 (pRW1)
Applied protein (mg)	17.4	18.6	19.5
Total activity applied (U)	56.8	130.7	2,4
Specific activity (U/g protein)	3260	7025	120
Total activity recovered (U)	4.8	26.6	0.35
Recovery (%)	8.5	20.4	14.5
Highest specific activity (U/g protein)	2360	8495	98

As described for DbfB and other extradiol dioxygenases, Edo2-2, 2-3 and 3 were highly unstable and lost between 25 and 50 % of activity per day when stored at 4 °C. Storage at -70 ^{0}C resulted only in a low loss of activity (less than 10 % per day). Partially purified enzymes, when purified under aerobic conditions, were highly unstable and complete loss of activity was usually observed in between 24 hours. In

order to, despite the instability of the proteins, get indications for their kinetic properties, K_m values and relative maximal transformation rates with different dihydroxysubstituted substrates were recorded with freshly prepared cell free extracts and eluates after MonoQ chromatography containing highly active extradiol dioxygenases Edo2-2 and Edo2-3.

Table 3.4. Kinetic constants were determined as described in 2.6.6. Maximal turnover rates of 3-methylcatechol by a given enzyme preparation are given relative to those measured with 2,3-dihydroxybiphenyl as substrate.

Substrate		**Edo2-2**		**Edo2-3**	
		K_m (µM)	V_{max} (%)	K_m(µM)	V_{max} (%)
2,3-dihydroxybiphenyl	Cell extract	140A25	100	150A40	100
	MonoQ eluate	95A20	100	80A25	100
3-methylatechol	Cell extract	410A80	35	520A130	45
	MonoQ eluate	455A30	35	370A80	55

Both Edo2-2 and Edo2-3 showed K_m-values with 3-methylcatechol of around 400–500 µM, independently if the kinetic data were measured using cell extract or partially purified enzyme, and maximal turnover rate was always one third to half of that measured with 2,3-dihydroxybiphenyl. K_m values determined in cell extracts were slightly lower than those determined with partially purified enzyme. As thus no major differences were observed in kinetic data using cell free extract versus partially purified protein, further kinetic constants of Edo2-2 and Edo2-3 as well as of Edo3 were measured in cell extract and compared to those reported for DbfB of RW1. The results are summarized in table 3.5.

Tab. 3.5: Catalytic properties of different 2,3-dihydroxybiphenyl 1,2-dioxygenases originating from *Sphingomonas* sp. RW1.

The kinetic parameters were determined using cell extracts of *E. coli* BL21(DE3)[LysS] expressing Edo 2-2, 2-3 and 3, respectively and compared to those described for DbfB by Happe et al. (Happe *et al*., 1993). K_m values are expressed in µM for all substrates. Maximal turnover rates are expressed relative to those determined with 2,3-dihydroxybiphenyl as substrate. The specific maximal activity expressed as U/mg of protein appears in parentheses. As turnover numbers were not calculated, the V_{max} /K_m values were compared relative to that for 2,3-dihydroxybiphenyl set as 100, as indication for the specificity constant K_{cat}/K_m.

Substrate	**Edo 2-2**			**Edo 2-3**			**Edo 3**			**DbfB**		
	K_m (µM)	V_{max} (%)	V_{max} / K_m (%)	K_m (µM)	V_{max} (%)	V_{max} / K_m (%)	K_m (µM)	V_{max} (%)	V_{max} / K_m (%)	K_m (µM)	V_{max} (%)	V_{max} / K_m (%)
2,3-dihydroxybiphenyl	140 A25	100	100 (6.5)	150 A40	100 (18.7)	100	55 A5	100 (2.3)	100	8 A2	100 (9.7)	100
catechol	830 A270	30	5	725 A120	33	7	4000 A900	3	0.04	83000 A4000	42	0.004
3-methylcatechol	410 A80	35	12	520 A130	45	13	10500 A1500	26	0.14	5300 A600	29	0.04
2,2‘,3-trihydroxybiphenyl	40 A15	28	98	65 A15	46	106	30 A5	90	165	11 A1	30	22

No major differences in terms of substrate preferences could be observed between Edo2-2 and Edo2-3. Both enzymes exhibit higher K_m values with the monocyclic dihydroxylated compounds tested than with the tested bicyclic substrates. Taken the V_{max}/ K_m value as an indication for substrate specificity, 2,3-dihydroxybiphenyl and 2,2‘,3-trihydroxybiphenyl were preferred over catechol and 3-methylcatechol as substrates. Similarly, 2,3-dihydroxybiphenyl and 2,2‘,3-trihydroxybiphenyl were the preferred substrates also for Edo 3 and DbfB. However those enzymes had significantly higher K_m values with monocyclic substrates tested when compared to Edo2-2 or 2-3. The 2,3-dihydroxybiphenyl can be regarded as a 1000-fold better substrate than the monocyclic substrates in case of Edo 3 and DbfB, whereas in case of Edo 2-2 and 2-3 the 2,3-dihydroxybiphenyl was only 10-20 better substrate. Comparison of 2,3-dihydroxybiphenyl and 2,2’,3-trihydroxybiphenyl as substrates between the enzymes showed that Edo 3 obviously has a slight preference for the trihydroxybiphenyl, while DbfB extradiol dioxygenase had a slight preference for the dihydroxybiphenyl.

3.3 Biochemistry of the 2,2',3-trihydroxybiphenyl ether metabolism

3.3.1 Analysis of the transformation products on HPLC

The extradiol cleavage of 2,2',3-trihydroxybiphenyl is supposed to result in the formation of the 6-(2-hydroxyphenyl)ester of 2-hydroxymuconic acid, which by spontaneous hydrolysis, gives rise to catechol and 2-hydroxymuconate (Wittich *et al*., 1992) (Wittich personal communication). Thus appearance of a yellow color in transformation experiments with 2,2',3-trihydroxybiphenyl as substrate is not a measure for substrate transformation but only for transformation of the product catechol by the enzyme. In initial experiments the transformation of 2,2',3-trihydroxybiphenyl ether (THBE) was thus analyzed by monitoring the oxygen consumption during the dioxygenase reactions with an oxygen electrode as described by Happe (Happe *et al*., 1993). Like for DbfB, 2,2',3-trihydroxybiphenyl ether dependent oxygen uptake was observed for all extradiol dioxygenases tested: Edo2-2, Edo2-3 and Edo3. However, this activity rapidly declined in between seconds indicating an inactivation of the enzyme during substrate turnover. Addition of 2,3-dihydroxybiphenyl to enzyme assays previously incubated with 2,2',3-trihydroxybiphenyl ether showed, that the enzyme was actually inactivated, as neither oxygen uptake nor the formation of a yellow color

appeared in the photometric assay. To verify that all extradiol dioxygenases of RW1 are capable to transform 2,2',3-trihydroxybiphenyl ether, a volume of 1 ml crude extract in phosphate buffer pH 7.5 was obtained from 50 ml culture, respectively, of *E. coli* BL21(DE3)[LysS](pT7-22), *E. coli* BL21(DE3)[LysS](pT7-23), *E. coli* BL21(DE3)[LysS](pT7W4) and *E. coli* BL21(DE3)[LysS](pT7-5-RW) cells cultured as previously described (2.6.2). A quantity of about 50 µg total proteins from each crude extract preparation was incubated with 2,2',3-trihydroxybiphenyl ether at a final concentration of 200 µM and in a total volume of 1 ml 50 mM potassium phosphate buffer pH 7.6 over 30 minutes. The 2,2',3-trihydroxybiphenyl-ether metabolites were analyzed by HPLC. An aqueous solvent system containing 40 % of methanol and 0.1 % *ortho*-phosphoric acid at a flow rate of 1 ml per min was used, which allowed the elution of all above described metabolites in about 17 minutes detection time. Samples were taken from the reaction solution at times 0, 15 and 30 minutes and immediately injected in HPLC (fig. 3.9). Due to the rapid inactivation process, the transformation products appeared only in traces however in sufficient amount to be detected after by HPLC analysis. All enzymes tested were capable to transform 2,2',3-trihydroxybiphenyl ether as evidenced by the formation of two reaction products, which were identified as catechol and 2-pyrone-6-carboxylate by comparison of their retention behavior with those of authentic standards. Furthermore, the absorption spectra with maxima at 210 nm and 270 nm on one hand and 200nm and 300 nm on the other were identical to those of authentic catechol and 2-pyrone-6-carboxylate, respectively. The formation of catechol and 2-pyrone-6-carboxylate with an absorption maximum at 300 nm at neutral conditions ($\varepsilon = 8.700\ M^{-1}\ cm^{-1}$) from 2,2',3-trihydroxybiphenyl ether contrasts previous assumptions of spontaneous rearrangement of the ring-cleavage product into catechol and 2-hydroxymuconate. No appearance of 2-hydroxymuconate could be observed.

The formation of 2-pyrone-6-carboxylate opened up the possibility to develop a simple enzymatic test for 2,2',3-trihydroxybiphenyl ether transformation based on the formation of 2-pyrone-6-carboxylate. At a wavelength of 290 nm, the absorption of catechol, as well as its ring cleavage product 2-hydroxymuconic semialdehyde, or the 2,2',3-trihydroxybiphenyl ether, are negligible ($\varepsilon < 1000\ M^{-1}\ cm^{-1}$) compared to that of 2-pyrone-6-carboxylate ($\varepsilon = 7.800\ M^{-1}\ cm^{-1}$). Thus, transformation of 2,2',3-trihydroxybiphenyl ether was followed at 290 nm..

A clear rise in absorption could be monitored using 10–100 µM 2,2',3-trihydroxybiphenyl ether and Edo 2-2 containing cell extract corresponding to a final

concentration of 20–100 µg/ml. Activity usually declined significantly in between 20 seconds. The activity, measured as the increase in absorbance at 290 nm during the first 2-5 seconds of reaction, was clearly proportional to the amount of enzyme added. However, determinations of activities at different substrate concentration were not accurate enough to determine kinetic parameters for the turnover of this substrate. It could however be calculated that activity of Edo 2-2 with 20 µM of 2,2',3-trihydroxybiphenyl ether was 65% of that with 20 µM 2,3-dihydroxybiphenyl.

To get an indication on the affinity of Edo 2-2 for 2,2',3-trihydroxybiphenyl ether, the rate of DHB transformation (DHB was added to the reaction mixture in a concentration of 100 µM) was quantified photometrically (434 nm) in the presence of varying amounts of substrate (20-200 µM THBE). Clearly, the addition of 2,2',3-trihydroxybiphenyl ether inhibited DHB transformation. This inhibition was evident even in the first seconds of the reaction and thus different from the enzyme inactivation observed during 2,2',3-trihydroxybiphenyl ether transformation. Assuming competitive inhibition to take place in the first seconds, the inhibition constant K_i of 2,2',3-trihydroxybiphenyl ether can be assumed to be in the range 40-80 µM. It seems thus, that the enzyme has a reasonable high affinity for this compound. As Edo 2-2 like the other extradiol dioxygenases tested is rapidly and completely inactivated during THBE turnover, a turnover number, i.e. the amount of substrate molecules one enzyme molecule can transform before being completely inactivated, could be a measure for the effectivity of the enzyme. As none of the enzymes was purified to homogeneity, turnover numbers could not be calculated. However, the turnover capacity of the different enzymes could be compared by calculating the amount of THBE that can be transformed by the amount of respective extradiol dioxygenase, which cleaves 1 µmol of 2,3-dihydroxybiphenyl/min (1U). The total amount of 2-pyrone-6-carboxylate produced until complete inactivation of Edo 2-2 was proportional to the amount of enzyme added. It could be calculated that the amount of Edo 2-2 containing cell extract that is capable to transform 1 µmol/min of 2,3-dihydroxybiphenyl (1U) transforms a total of 0.05 µmole of THBE. Similar experiments were performed using Edo 3 or DbfB containing cell extracts. The activity of Edo3 with 20 µM THBE was only 25 % of that with 20 µM DHB. 1U of Edo 3 was calculated to be capable to transform 0.04 µmole of THBE. Thus, Edo 3 seems to be less effective than Edo 2. No significant 2-pyron-6-carboxylate forming activity was observed with DbfB. This activity was less than 1% of that with DHB at substrate concentrations between 4 and 100 µM.

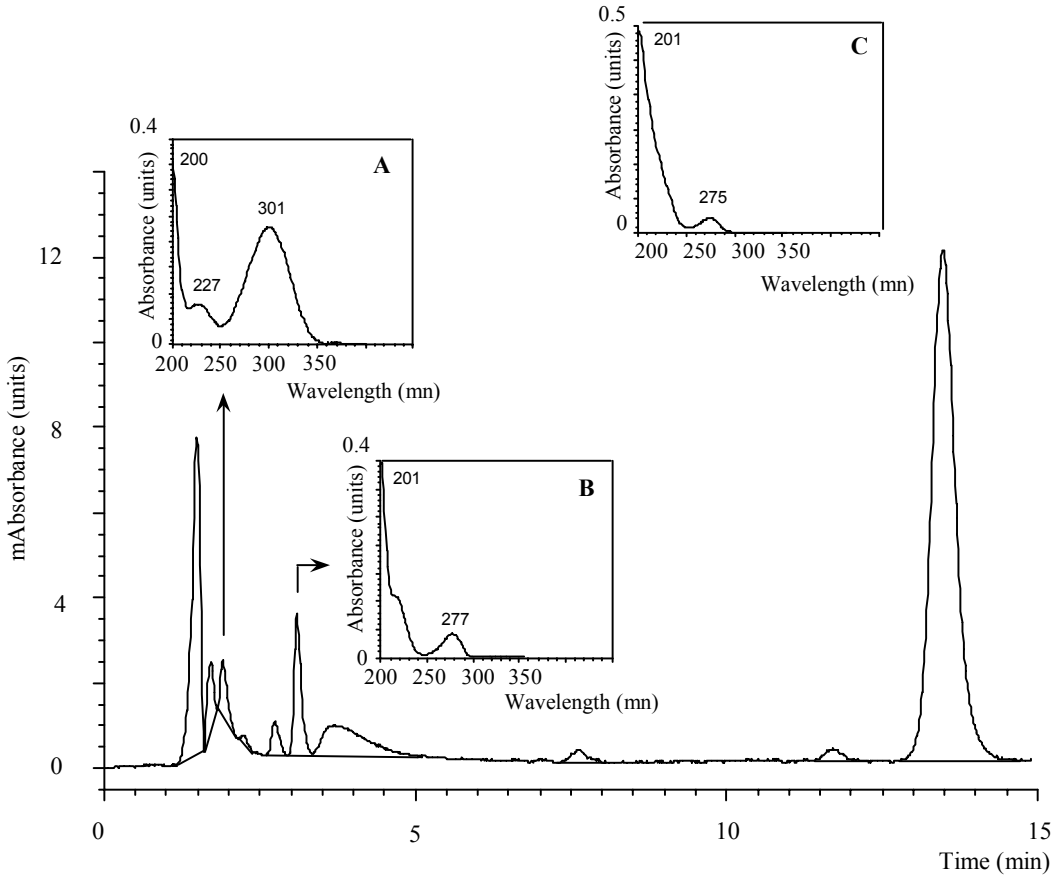


Fig. 3.9: Transformation of the 2,2',3-trihydroxybiphenyl ether by Edo2-3. The chromatogram was recorded at a wavelength of 210 nm and corresponds to a sample taken after 30 minutes incubation with the substrate. Absorption spectra indicated on the figure next to the corresponding peaks refer to the compounds: A: 2-pyrone-6-carboxylate; B: catechol; C: 2,2',3-trihydroxybiphenyl ether.

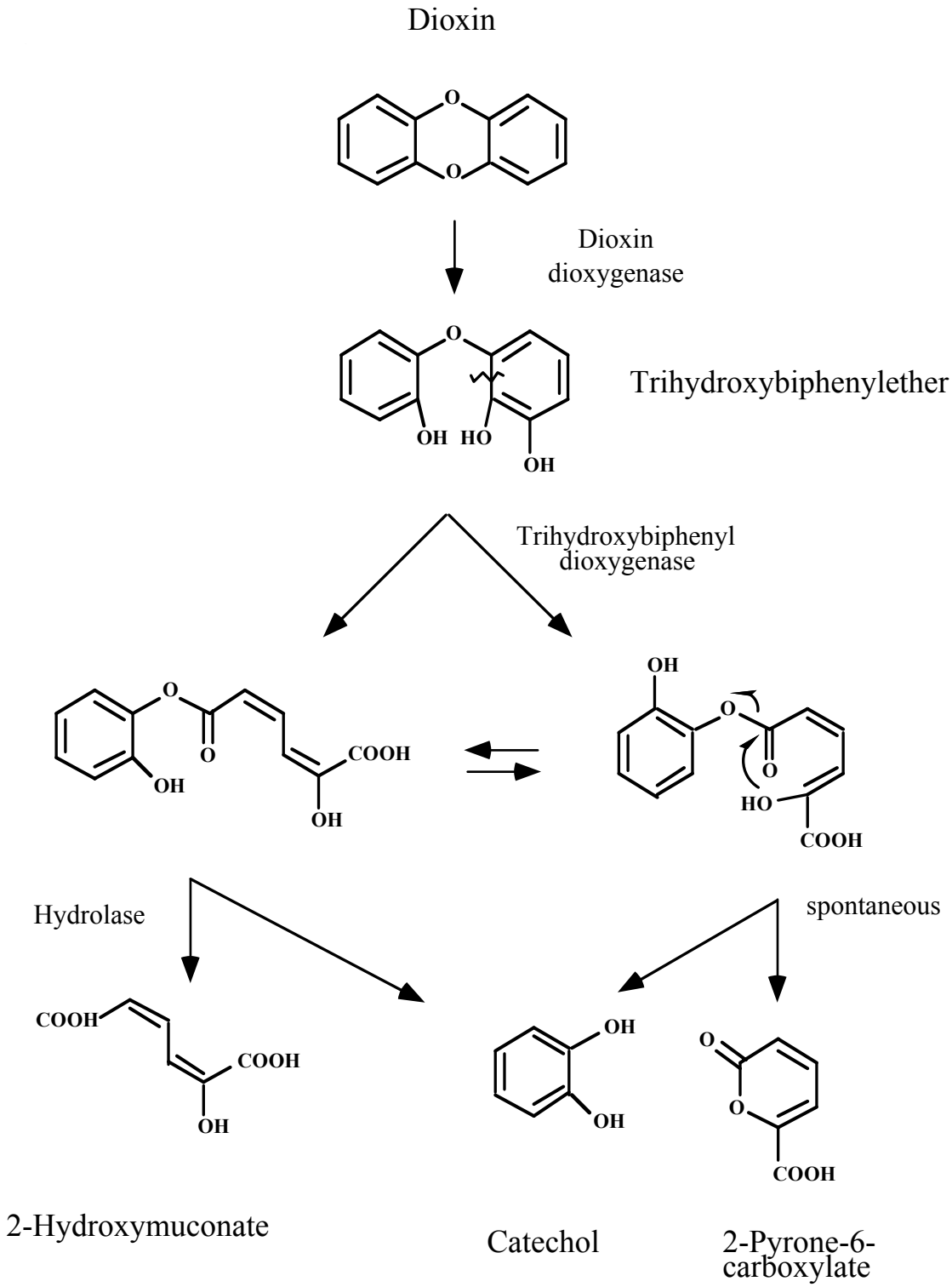


Fig: 3.10: Dioxin degradation upper-pathway deduced from the HPLC analysis.

3.3.2 Further investigation on the inhibition of RW1 extradiol dioxygenase, caused by the reaction substrate 2,2',3-trihydroxybiphenyl ether

The following experiment has been performed to better understand the mechanism through which 2,2',3-trihydroxybiphenyl ether inactivates the extradiol dioxygenases of *Sphingomonas* sp. RW1 in vitro. Studies have been conducted on Edo 2, in the form of Edo 2-3, having this enzyme a high affinity for the 2,2',3-trihydroxybiphenyl ether in comparison with the other RW1 extradiol dioxygenases. In particular Edo2-3 was used instead of Edo2-2, as it was more stable in crude extract at room temperature but was undistinguishable from Edo2-2 concerning the kinetic parameters. A volume of 2 ml crude extract was prepared from 50 ml *E. coli* BL21(DE3)[LysS] (pT7-23) culture, under anaerobic conditions as previously described (2.6.3). Half volume of the crude extract was aerobically stored and half anaerobically, in a 2ml Duran glass closed with caucciu sealing and filled up with N_2. Both preparations were kept cool on ice. A 1:10 dilution in 50 mM potassium phosphate buffer (pH 7.5) of both anaerobic and aerobic crude extracts was prepared. The activity of the diluted extracts was measured with 1 mM 2,3-dihydroxybiphenyl as substrate using 10 µl protein preparation in a final reaction volume of 1 ml 50 mM potassium phosphate buffer (pH 7.5) and following the appearance of the *meta*-cleavage product HOPDA (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid) during the first seconds of reaction (2.6.6). A specific activity of 3 U/g and 0.8 U/g could be registered with the aerobic and anaerobic diluted protein extract preparation respectively. The diluted aerobic and anaerobic protein preparations were then incubated with a final concentration of 2 mM 2,2',3-trihydroxybiphenyl ether for 5 minutes. An anaerobic 2,2',3-THB ether solution (2.2.1) was especially set up to be added to the diluted anaerobic Edo2-3 protein solution. After the incubation period, the residual activity in 10 µl solution Edo2-3 plus THBE was monitored with DHB as substrate (20 µM THBE in the reaction volume). No residual *meta*-cleavage activity with 2,3-DHB was detectable with the aerobic preparation. On the opposite, a specific activity of 0.6 U/g could be still recorded with the diluted cell extract anaerobically incubated in the presence of THBE, during the first seconds of reaction and before occurrence of inactivation, following exposure of the reaction mixture to O_2 during the performance of the enzymatic assay. It can thus be concluded that 2,2',3-trihydroxybiphenyl ether itself is not responsible for the inactivation of the extradiol dioxygenase Edo2-3, but that inactivation needs the presence of oxygen and thus the

turnover of the substrate. As additional control Edo2-3 was incubated with 2 mM 2-pyrone-6-carboxylate, being this one of the 2,2',3-trihydroxybiphenyl ether transformation products. No inhibition in this case has been observed, excluding the hypothesis that the compound 2-pyrone-6-carboxylate could be responsible of the inactivation of the *meta*-cleavage enzymes.

3.4 Realization of two compact modular genetic cassettes encoding the dibenzofuran and dioxin degradation upper-pathways

As all elements of the catabolic upper pathway for dibenzo-*p*-dioxin and dibenzofuran degradation have been described, the goal was to transfer those genes finally into organisms which are capable to handle chlorinated derivatives of catechol and salicylate, respectively and thereby to develop microorganisms capable to mineralize chlorinated derivatives of dibenzofuran and dibenzo-*p*-dioxin. For doing this, the genetic elements comprising the upper pathway have to be reassembled into compact modular gene cassettes. Those cassettes furthermore should be constructed in a fashion enabling the rational exchange of a certain gene by another one encoding for a protein of similar function, thus allowing the analysis of the function of various members of a family of genes in the degradation of a certain substrate. In the present work, two different genetic cassettes, addressed respectively to dioxin and dibenzofuran degradation, were constructed and analyzed. For the degradation of dibenzofuran into salicylate and of dibenzo-*p*-dioxin into catechol and 2-pyron-6-carboxylate, at least three enzyme systems are necessary, the initial angular dioxin dioxygenase system, a *meta*-cleavage dioxygenase and a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate-type hydrolase (fig. 1.2). As previously described (Armengaud *et al*., 1998), the respective genes were dispersed over the chromosome of *Sphingomonas* RW1 strain. Moreover, genetic analysis of RW1 revealed the presence of a variety of genes obviously coding enzymes of a similar function, such as the three extradiol dioxygenases mentioned above. Both genetic cassettes (fig. 3.12, 3.13) contain as the first three genetic determinants the genes *dxnA1A2, fdx1* and *redA2* coding for the angular dioxin dioxygenase system, consisting of the DxnA1A2 dioxygenase, the ferredoxin Fdx1 and the reductase RedA2, respectively. The two expression modules differ in the gene encoding the *meta*-cleavage enzyme. The gene *dbfB*, encoding trihydroxybiphenyl

dioxygenase, was chosen for the realization of the genetic cassette addressed to the DF degradation, while the gene *edo2-2*, coding for the *Sphingomonas* RW1 Edo2-2 extradiol dioxygenase, exhibiting the most favourable kinetic parameters for 2,2',3-trihydroxybiphenyl ether transformation (3.3), was included in the cassette suitable for DD degradation. Several 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolases have been characterized in *Sphingomonas* sp. RW1 (Armengaud *et al*., 1998; Bunz *et al*., 1993). Among them, DxnB was shown in our laboratory to convert hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoic acid with the respective highest activity. Furthermore *dxnB* gene clusters with the genes encoding the ring-hydroxylating dioxin dioxygenase (Armengaud *et al*., 1998). and is thus possibly the enzyme predominantly involved in both DF and DD degradation pathways. Therefore *dxnB* has been included in both cassettes. To reassemble all these ORFs, rare recognition restriction sequences, 8 bp in length and corresponding to the restriction enzymes: *Swa*I, *Asc*I, *Pac*I, *Pme*I and *Sse*8387I, were introduced by PCR amplification, flanking the 5' and 3' ends of each gene of the expression modules. Two 6 bp restriction sites, *Spe*I and *Bsi*WI, were additionally introduced upstream and downstream of the expression modules, respectively.

The resulting two compact expression modules are *dxnA1A2_redA2_fdx1_dbfB_dxnB* and *dxnA1A2_redA2_fdx1_edo2-2_dxnB* respectively. Both cassettes were tested for conversion of dibenzofuran and dioxin in *E. coli* DH10B.

Table 3.6: Primers used for the amplification of the elements of the expression modules *dxnA1A2_redA2_fdx1_dbfB_dxnB* and *dxnA1A2_redA2_fdx1_Edo2-2_dxnB*.

Primer	Amplified genes	Primer sequences
M40 forward	*dxnA1A2*	GGATTTAAATGGACTAGTGTTTGCTCAATTCAGTCGGGCG
M27 reverse	*dxnA1A2*	GCGCCAGATCTGTTGAGCTCACTGGGTG
M41 reverse	*dxnA1A2*	ATGGCGCGCCGTCAGTTCAATTATCCAATAATATTCC
M28 forward	*dxnA1A2*	AACAGATCTGGCGCATCTGCGCGCCAGTTGCCGAAAATCAG
M42 forward	*fdx1-redA2*	GGGGCGCGCCGGAAGAGAAGGAAAGTAGG
M43 reverse	*fdx1-redA2*	CGGTTAATTAACGCTACGGTACAAGGCCGATTTC
M44 forward	*dbfB*	GGGTTAATTAAGGAGCAAAAGGAGAGTTATCG
M45 reverse	*dbfB*	CGGTTTAAACGACAATGTTAGGTGTATG
M47 forward	*H1*	GGGTTTAAACGTCGCCCGCGAAGTGAGGATAG
M46 reverse	*H1*	GCCCTGCAGGCGTACGGTCAAACGGATCGGAACTCG
M32 forward	*Edo2-2*	GCTTAATTAACTCGCCTATCCGCTTTG
M34 reverse	*Edo2-2*	CTGTTTAAACGCCCTCCGCGCACAC
M33 forward	*Edo2-3*	CTGTTTAAACGCCCTCCGCGCACAC

3.4.1 Amplification of the different elements to be assembled in the expression modules

The templates used for the amplification of the genes *dxnA1A2* and *dxnB, dbfB, redA2* together with *fdx1*, and *Edo2,* were cosmid pAJ115, cosmid pAJ114, plasmid pAJ130 (Armengaud *et al*., 1998) and plasmid pRW1, respectively. The genes were amplified comprising of at least 20 bp upstream of their ATG starting codons including therefore the putative Shine Dalgarno sequences. The primers used for this purpose were designed in a way to introduce unique restriction sites flanking each genetic element of the cassette (table 3.6).

The unique restriction sites are mostly rare 8 bp cutters and are not present in the sequence of any of the genes included in the two cassettes. The only exception was as *Asc*I restriction site which was found at position 459 downstream of the ATG in

dxnA1A2. It was therefore necessary to eliminate the internal *Asc*I site, which was done through PCR as follows.

The PCR amplification of *dxnA1A2* was carried out in two steps. First, primers M40 and M27 were used to amplify a 542 bp fragment corresponding to the 5' part of the gene: M40 primer introduced the restriction sites *Swa*I and *Spe*I at the 5' end of the amplified fragment, while M27 primer eliminated, by mean of a point mutation, the *Asc*I site and introduced a new *Bgl*II site at its 3' end, to be used for the subsequent reconstitution of the gene. Primers M41 and M28 were used to amplify the 1466 bp fragment corresponding to the 3' end of *dxnA1A2* gene. Primer M41 introduced the restriction site *Asc*I, necessary for the assembling of the genetic cassette, at the 5' end of the amplified fragment and necessary for the assembling of the genetic cassette, while primer M27 was designed to complement the *Bgl*II site introduced by primer M27 (fig. 3.11). None of the point mutation introduced by PCR to create the *Bgl*II site or abolish the *Asc*I site, modified the protein aminoacid sequence. The 542 and 1466 bp amplified blunt end fragments were ligated to plasmid pCR-Script Amp SK(+) precut with *Srf*I originating the recombinant plasmids pMDKA and pMDKB respectively. Genes *redA2* and *fdx1* could be coamplified in a single fragment by using as a template plasmid pAJ130 realized by J. Armengaud (Armengaud *et al*., 1998). The 1691 bp fragment carrying both genes *redA2* and *fdx1* was amplified by mean of primers M42 and M43, introducing an *Asc*I site and a *Pac*I site at the 5´and 3´ ends of the amplified fragment respectively. The PCR product was inserted into pCR2.1 resulting in plasmid pMDK3. The amplification of the extradiol dioxygenase gene *dbfB* was carried out with primers M44 and M45, introducing a *Pac*I and a *Pme*I restriction sites at the 5' and 3' ends of the fragments respectively. The amplified 957 bp fragment was purified and inserted into pCR-Script Amp SK(+) resulting in pMDK1. The amplification of the hydrolase *dxnB* gene was carried out with primers M45 and M46, introducing a *Pme*I restriction site and the two sites *Bsi*WI and *Sse8387*I at the 5' and 3' ends of this gene, respectively. The amplified 923 bp fragment was purified and inserted into pCR2.1 resulting in pMDK4. The amplification of *Edo2-2*, was carried out with primers M32 and M34 introducing a *Pac*I and a *Pme*I restriction sites at the 5' and 3' ends of the fragment, respectively. The resulting 1032 bp fragment was purified and inserted into pCR-Script Amp SK(+) resulting in pMDK7. All the inserts of the pMDKA, pMDKB, pMDK3, pMDK1 and pMDK7 plasmids were sequenced on both strands in order to check the integrity of the nucleic information.

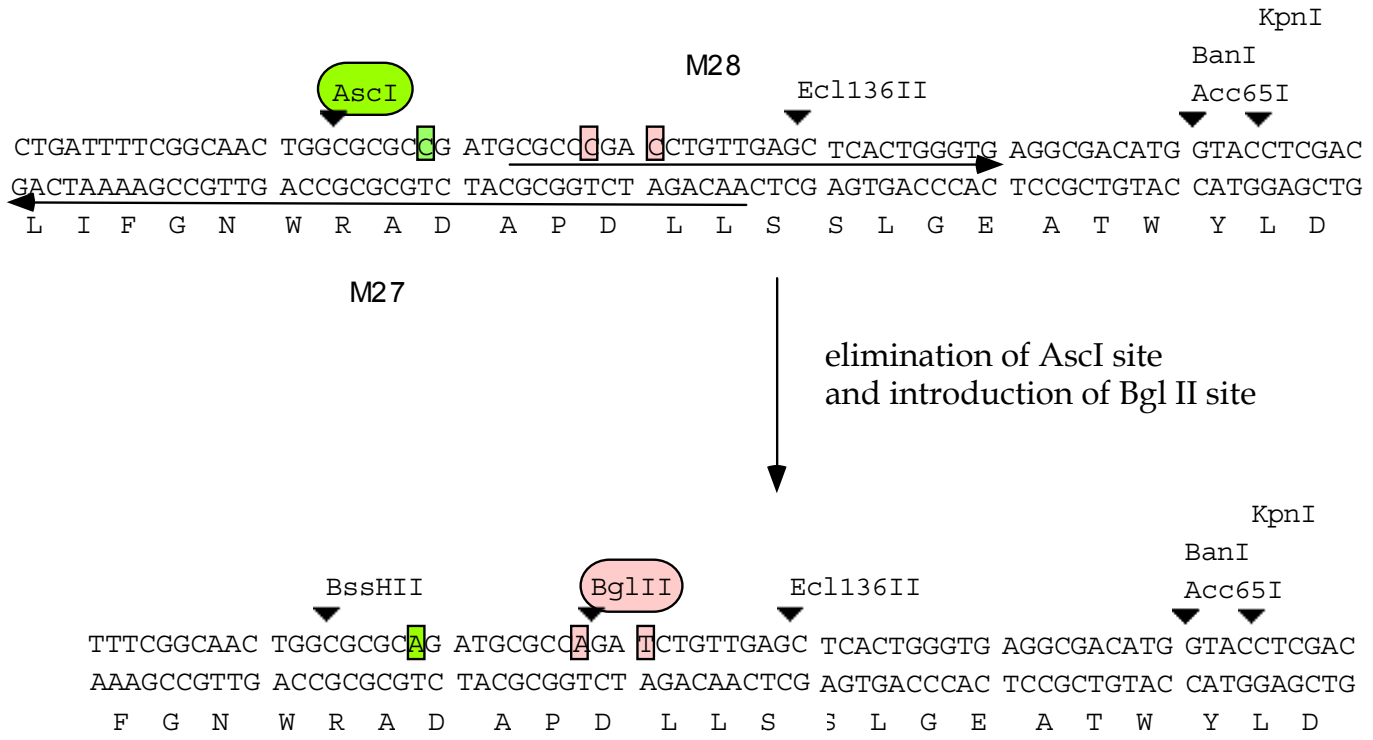


Fig 3.11: Site-directed mutagenesis of *dxnA1A2* gene carried out by PCR eliminating an *Asc*I site and introducing a *Bgl*II site.

3.4.2 Assembling of the expression module *dxnA1A2_redA2_fdx1_dbfB_dxnB* and construction of the expression vector pMDK12

As initial step in the realization of the modular cassettes, *dxnA1A2* gene was initially reconstituted in pNEB193, as follows. Plasmid pMDKA was subjected to digestion by *Sac*I and *Bgl*II originating a 563 bp fragment. The *Sac*I restriction site originally belongs to the MCS of vector pCRScript in construct pMDKA. Since it does not cut the insert and is present in the MCS on pNEB193 as well, *Sac*I was used to transfer the 542 bp insert from pMDKA to pNEB193. *Swa*I and *Spe*I sites, supposed to flank the 5' end of the genetic cassette, and introduced by PCR at the 5' region of the 542 bp insert (3.4.1), are obviously also included in the 563 bp fragment resulting from the *Sac*I-*Bgl*II digestion of pMDKA, and directly follow *Sac*I site. Concomitantly, plasmid pMDKB was subjected to digestion by *Bgl*II and *Asc*I. The resulting 1459 bp *Bgl*II -*Asc*I and 563 bp *Sac*I-*Bgl*II fragments were purified and ligated into vector pNEB193 previously digested by *Sac*I and *Asc*I, originating the construct pMDK2. In a similar way, the two inserts from pMDK1 and pMDK4 were excised by digestion with *Pac*I and *Pme*I, and, *Pme*I and *Sse8387*I, respectively. After purification, the resulting 952 bp and 933 bp fragments were inserted into vector pNEB193 previously subjected to restriction with *Pac*I and *Sse8387*I. The resulting construct, pMDK8 was then digested with *Pac*I and *Sse8387*I, while pMDK3 was digested with *Asc*I and *Pac*I. The two resulting fragments, respectively 1877 bp and 1686 bp in length, were purified and inserted into pMDK2 already digested with *Asc*I and *Sse8387*I. The resulting construct, designated pMDK10, was checked by several restriction analyses. Plasmid pMDK10 was digested with *SwaI* and *Sse8387*I. The resulting 5580 bp fragment, carrying the complete expression module *dxnA1A2_redA2_fdx1_dbfB_dxnB*, was purified and ligated for expression purposes under the control of the Ptac promoter to vector pVLT35 previously subjected to *Sma*I and *Pst*I digestion, resulting in pMDK12 (fig. 3.12).

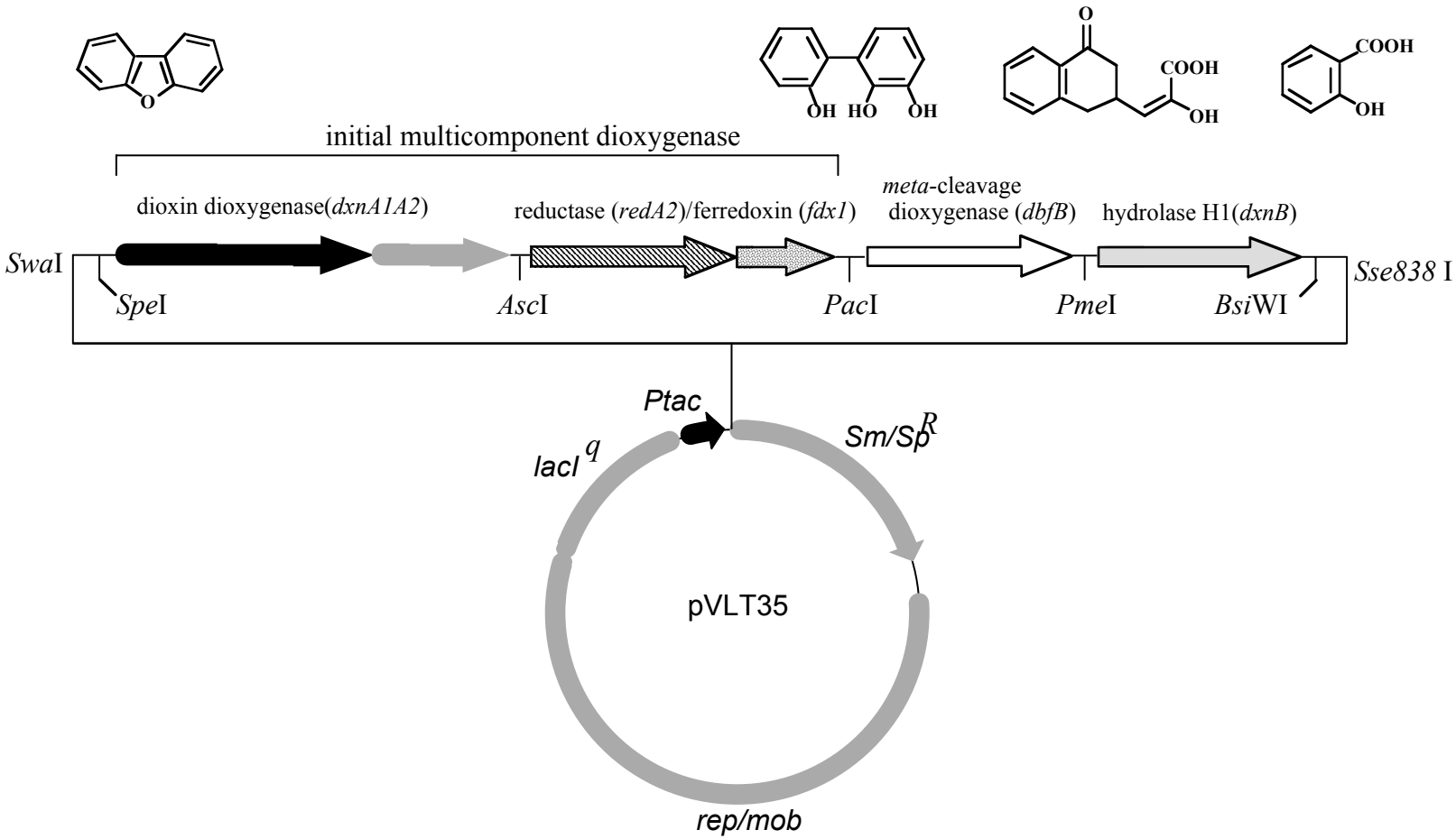


Fig. 3.12: Plasmid pMDK12. The expression module *dxnA1A2_red A2_fdx1_ dbfB_dxnB*. is ligated to vector pVLT35 under the control of the Ptac promoter. Unique restriction sites flanking each element of the cassette are indicated. The molecules corresponds to the main dibenzofuran metabolites originating from the activity of the enzymes codified by the cassette.

3.4.3 Realization of the expression module *dxnA1A2_redA2_fdx1_edo2-2_dxnB* and construction of the expression vector pMDK19.

The gene *dbfB* in the construct pMDK10 was substituted by the extradiol dioxygenase gene *edo2-2* (939 bp) as follows. Plasmid pMDK7 was digested with *Pac*I and *Pme*I. The resulting 1027 bp was purified and ligated with the 7274 bp fragment, originated from pMDK10 double digested with *Pac*I and *Pme*I, thus lacking the *dbfB* gene and carrying *dxnA1A2-redA2-fdx1* and *dxnB* genes. The resulting construct pMDK18 was subjected to restriction with *Swa*I and *Sse8387*I giving a 5651 bp fragment which was purified and ligated under the control of the Ptac promoter to vector pVLT35, previously digested with *Sma*I and *Pst*I, resulting in the construct pMDK19 (fig. 3.13). The recombinant clones were checked by several restriction enzymes for carrying the complete expression module *dxnA1A2_redA2_fdx1_edo2-2_dxnB*.

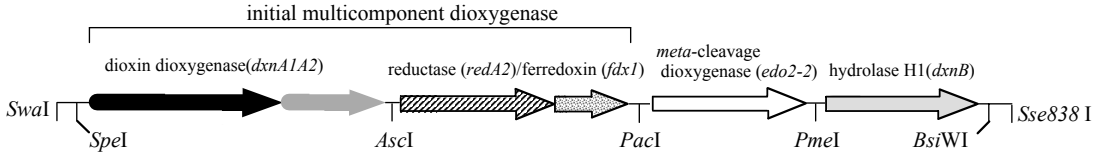


Fig. 3.13. Expression module *dxnA1A2_red A2_fdx1_ edo2-2_dxnB*. The extradiol dioxygenase gene *dbf*B has been substituted with *edo2-2*. The complete expression module has been cloned into vector pVLT35 under the control of the Ptac promoter.

3.5 Expression studies

3.5.1 Dibenzofuran conversion by *E. coli* (pMDK12)

The expression vector pMDK12 was transformed into *E. coli* DH10B by electroporation (2.5.9). The enzymes specified by the *dnxA1A2, redA2, fdx1, dbfB* and *dxnB* genes on pMDK12 were tested for their ability to transform dibenzofuran by incubating resting *E. coli* DH10B (pMDK12) cells at 30 °C with 0.1 mM of the substrate as described in 2.7.1 and following the reaction (i.e. disappearance of the substrate and formation of the products) over time by HPLC (2.7.2). Simultaneously, *E coli* DH10B (pMDK12) was tested for the capability to transform the expected reaction product salicylate. There was

no transformation of salicylate during a 24h incubation with resting cells of an OD_{600} of 10. Trihydroxybiphenyl (100 µM) was rapidly transformed by *E.coli* DH10B (pMDK12) into 3-(chroman-4-on-2-yl)-pyruvate (approximately 90 µM) due to the relatively high *meta*-cleavage activity and spontaneous rearrangement of the ring-cleavage product 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoate. Only minor amounts of salicylate (6-9 µM) were formed in this case. 3-(Chroman-4-on-2-yl)-pyruvate can regarded to be relatively stable during an incubation period of 8 hours but minor disappearance of this compound was observed during extended incubation. Similar results were obtained when tryhydroxybiphenyl (100 µM) was subjected to transformation by a cell free extract of *E. coli* DH10B (pMDK12).

The activity of DbfB in those extracts of *E. coli* DH10B (pMDK12) was quantified as a high 250-300 U/g protein with dihydroxybiphenyl as substrate. Activity of DxnB was quantified by the rate of formation of benzoate by *E. coli* DH10B (pMDK12). The formation of benzoate as quantified by HPLC occurred at rates of 3-5 µM/min when cells of an optical density of 5 were applied. This corresponds, assuming an OD_{600} of 1 to be indicative for a protein content of approximately 100 mg of protein/l, to an activity of 6-10 U/g protein. Thus, the hydrolase activity was significantly lower compared to the *meta*-cleavage activity, explaining mainly 3-(chroman-4-on-2-yl)-pyruvate formation and negligible salicylate formation during trihydroxybiphenyl transformation. Dibenzofuran transformation, as quantified by reversed phase HPLC analysis (see fig. 3.14 and 15) occurred at a rate of 0,2-0,3 µM/min by resting cells of an OD_{600} of 10, indicative for a transformation rate of 0,2-0,3 U/g protein. Salicylate was observed as the major transformation product formed in a yield higher then 90%. Only minor amounts of 3-(chroman-4-on-2-yl)-pyruvate, were formed. 2-Hydroxypenta-2,4-dienoate, expected to be produced during the DxnB catalyzed hydrolysis of 2-hydroxy-6-oxo-6-2-(hydroxyphenyl)hexa-2,4-dienoate into salicylate is not indicated in fig. 3.14 as this compound was not detectable by the HPLC method employed.

2,2´,3-trihydroxybiphenyl was observed not to accumulate during dibenzofuran transformation, indicating the ring-cleavage not to be rate-limiting in this recombinant degradation pathway. Formation of mainly salicylate and only minor amounts of 3-(chroman-4-on-2-yl)pyruvate further indicates that, in contrasts to the situation with 2,2',3-trihydroxybiphenyl as substrate, hydrolysis by DxnB was not rate limiting during dibenzofuran transformation, showing that in *E. coli* DH10B (pMDK12) the initial angular dioxygenation is the limiting transformation reaction. From above evidence it

can be deduced that *E. coli* (pMDK12) follows the degradative route presented in fig. 3.16. The initial step of the degradation of DBF is a stereospecific angular dioxygenation of one of the aromatic rings, carried out by the dioxin dioxygenase (DxnA1A2) acting with a specific reductase (RedA2) and a ferredoxin (Fdx1). The reaction product spontaneously rearrange to 2,2´,3-trihydroxybiphenyl, subsequently *meta*-cleaved in the dihydroxylated ring by an extradiol dioxygenase, DbfB, producing 2,2´-dihydroxy-6-oxo-6-phenylhexa-2,4-dienoate.

The 2,2´-dihydroxy-6-oxo-6-phenylhexa-2,4-dienoate is then converted by an hydrolase into salicylate. In case of a significant accumulation of the 2,2´-dihydroxy-6-oxo-6-phenylhexa-2,4-dienoate, 3-(chroman-4-on-2-yl)-pyruvate will be formed by spontaneous rearrangement.

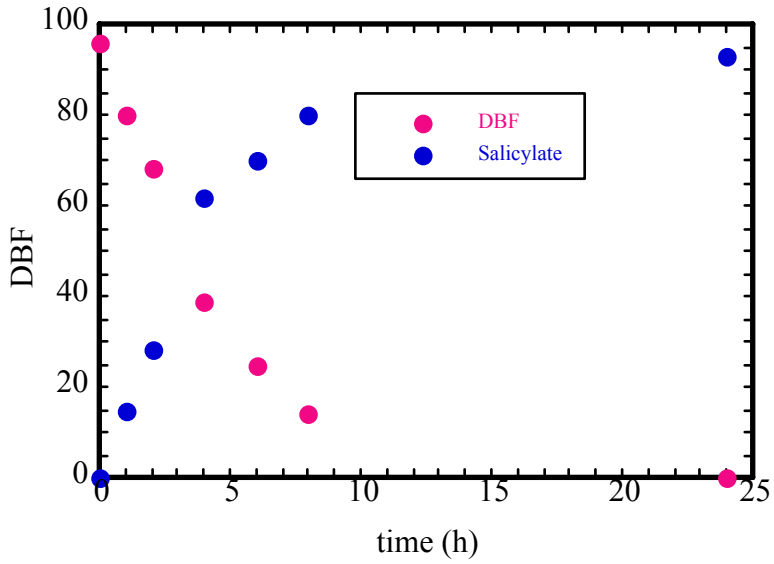


Fig. 3.14: Quantification over time of DBF transformation and salicylate accumulation by *E. coli* (pMDK12) cells of an OD_{600}.of 10. Product and substrate concentrations are expressed in µM.

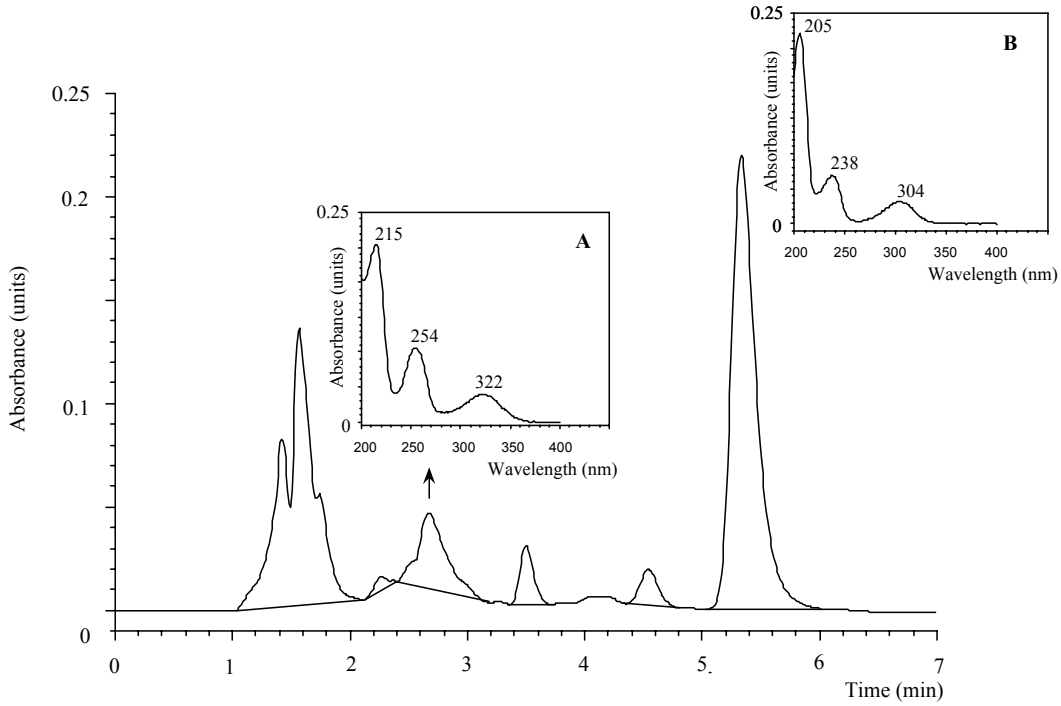


Fig. 3.15: End-products of the conversion of dibenzofuran by *E. coli* DH10B (pMDK12) as measured by HPLC. The chromatogram was recorded at a wavelength of 210 nm and shows analysis of culture supernatant collected after 24 h incubation with the substrate. Products between 1 and 2 minutes were produced also when *E. coli* was incubated without substrate and therefore are not transformation products. Absorption spectra are indicated for: A: 3(chroman-4-on-2-yl)-pyruvate and B: salicylate.

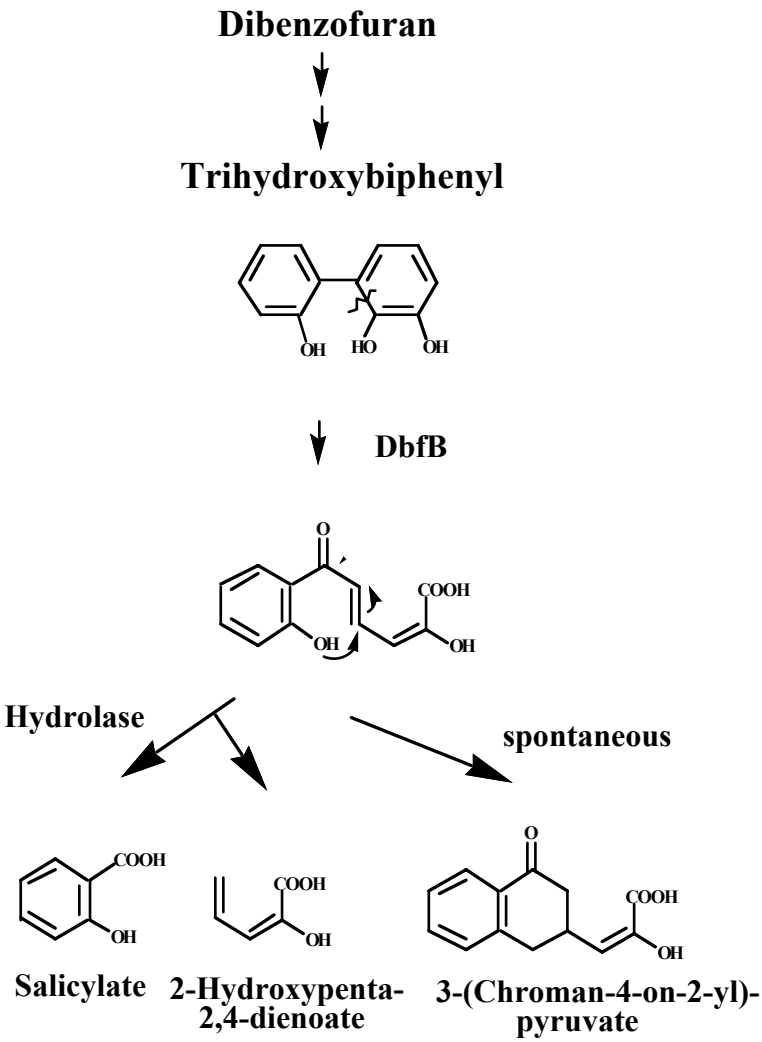


Fig 3.16: Dibenzofuran degradative route as it has been deduced from the HPLC analysis of the transformation products following incubation of *E. coli* DH10B (pMDK12) resting cells at 30 °C with 0.1 mM of dibenzofuran.

3.5.2 Dibenzofuran conversion by *E. coli* (pMDK19)

The expression vector pMDK19 was transformed into *E. coli* DH10B by electroporation. Like for *E coli* (pMDK12) also in the case of *E. coli* (pMDK19) experiments both in cell extract and with resting cells were performed. The activity of Edo2-2 expressed from pMDK19 was analyzed qualitatively by formation of a yellow colored ring-cleavage product during 2,3-dihydroxybiphenyl transformation by resting cells and quantified by use of a cell free extract. Induced cells of *E. coli* DH10B (pMDK19) exhibited relatively low, but significant activities of 20-25 U/g protein when measured with 100 µM of 2,3-dihydroxybiphenyl. In accordance with the relative activities observed with Edo2-2 overexpressed in *E. coli* (DE3)(LysS)(pT7-22), activity with trihydroxybiphenyl (50 µM) was 7-10 U/g protein, and that with catechol (100 µM) about 1.5 U/g protein. Thus the Edo2-2 gene product expressed from pMDK19 resembles in its kinetic properties that overexpressed in *E. coli* (DE3)(LysS)(pT7-22).

In accordance with results obtained with cell extracts, transformation of 2,3-dihydroxybiphenyl (500 µM), by resting cells of pMDK19 of an optical density of 5 occurred at rates higher than 15 µM/min, corresponding to activities >30 U/g protein and transformation of catechol at rates of 1-2 U/g protein, as quantified both by HPLC analysis of the catechol substrate as well as photometric analysis of the accumulation of the ring-cleavage product 2-hydroxymuconic semialdehyde. However, THB by resting cells was converted at rates significantly lower than those expected from experiments with cell free extracts. Whereas cell extract experiments, as stated above showed activities of 7-10 U/g protein, resting cells of an OD 5 transformed less than 20 % of a 100 µM solution of THB in 2 hours and thus exhibited activities lower than 0.5 U/g protein. The resting cells similarly to *E. coli* DH10B (pMDK12) were incubated at 30 °C with 0.1 mM dibenzofuran (2.7.1). Samples were taken at time points 0,1,2,4,6,8,24 hours and the transformation reaction followed by HPLC. Results are described in fig. 3.17.

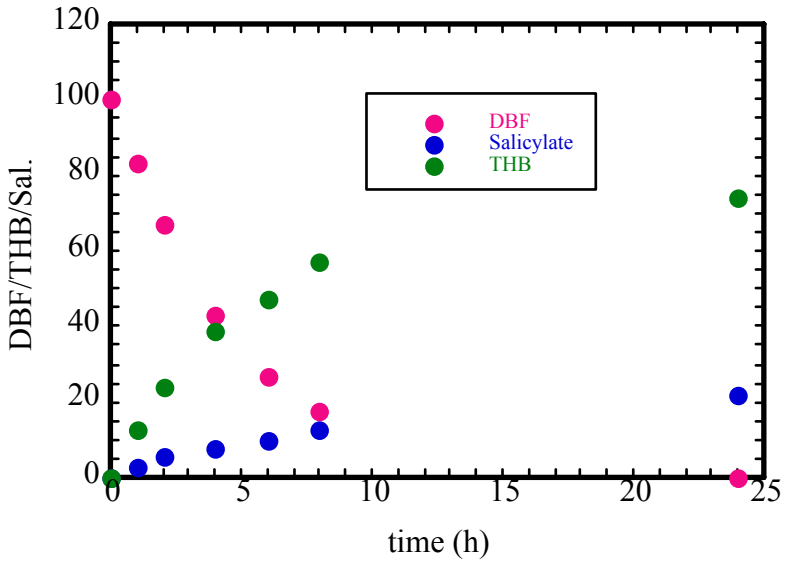


Fig 3.17: Quantification over time of DBF transformation and salicylate as well as trihydroxybiphenyl accumulation by *E. coli* (pMDK19) cells of an OD_{600} of 10. Product and substrate concentrations are expressed in µM.

Dibenzofuran transformation, as quantified by reversed-phase HPLC analysis (see fig. 3.14 and 15) occurred, like with pMDK12, at a rate of 0.2–0.3 µM by resting cells of an OD_{600} of 10, indicative for a transformation rate of 0.2–0.3 U/g protein. However, in contrast to pMDK12, both salicylate and trihydroxybiphenyl were observed as transformation products, together with minor amounts of 3(chroman-4-on-2-yl)-pyruvate. Whereas THB was formed at rates of about 0.2 U/g protein, salicylate formation occurred at rates lower than 0.05 U/g protein. Obviously, in pMDK19, ring-cleavage of THB rather than dioxygenation of dibenzofuran is the bottle-neck in the degradative pathway.

3.5.3 Transformation of dibenzo-*p*-dioxin by *E. coli* DH10B (pMDK12) and *E. coli* DH10B (pMDK19)

Cells of *E. coli* DH10B (pMDK12) and *E. coli* DH10B (pMDK19), carrying respectively *dbfB* and *edo2-2* genes, were tested for their ability to transform dibenzo-*p*-dioxin by incubation with 0.1 mM of this substrate and subsequent analysis of the transformation products by reversed-phase HPLC. In both cases, accumulation of the degradation intermediate 2,2',3-trihydroxydiphenyl-ether, resulting from the dihydroxylation of dibenzo-*p*-dioxin by the dioxin dioxygenase, and subsequent molecular rearrangement, was noticed. No other transformation products such as catechol or 2-pyrone-6-carboxylate (3.3.1) could be detected. Dioxin transformation, as quantified by reversed-phase HPLC analysis (fig.18) by both pMDK12 and 19 occurred at initial rates of 0.35–0.5 µM by resting cells of an OD_{600} of 10, indicative for a transformation rate of 0.35–0.5 U/g protein. Thus, dioxin transformation rates are significantly higher than dibenzofuran transformation rates.

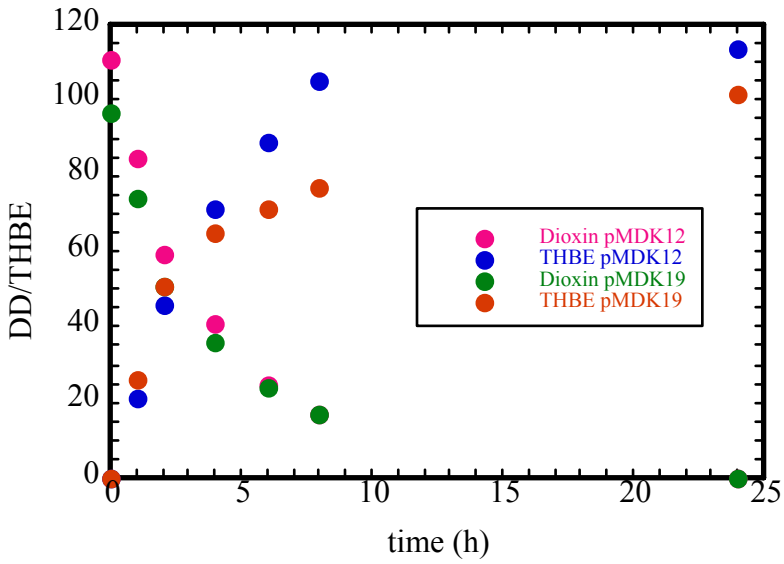


Fig. 18: Quantification over time of dioxin transformation and trihydroxybiphenyl ether accumulation by *E. coli* (pMDK19) or *E. coli* (pMDK12) cells of an OD_{600} of 10. Product and substrate concentrations are expressed in µM.

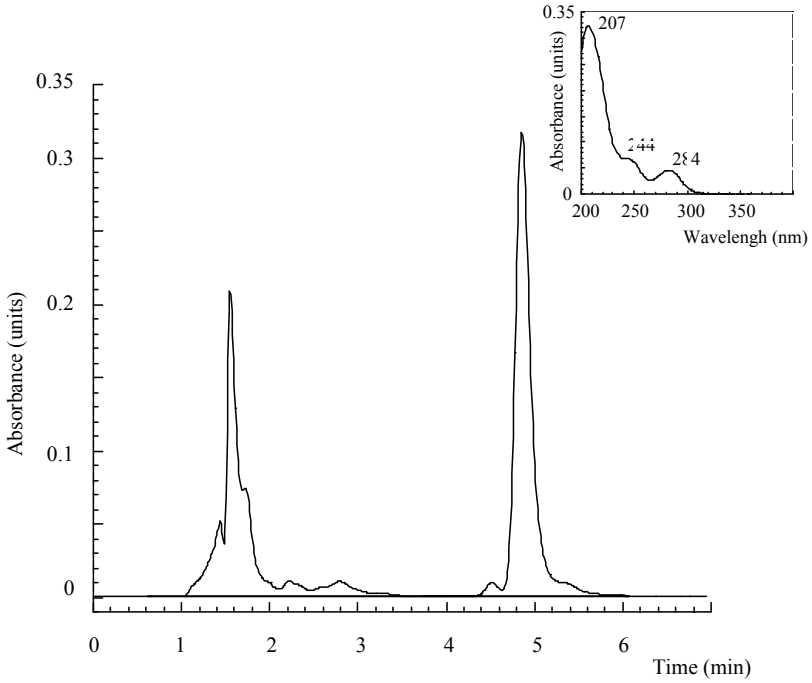


Fig 3.19. Conversion of dibenzo-*p*-dioxin by *E. coli* DH10B (pMDK19). The chromatogram was recorded at a wavelength of 210 nm and corresponds to a sample taken after 24 h incubation with the substrate. The only reaction product is the 2,2'.3-trihydroxybiphenyl ether for which the absorption spectrum is also shown.

3.5.4 Transformation of dibenzo-*p*-dioxin by mixed cell cultures

It has been previously described that all extradiol dioxygenases of RW1, are rapidly inactivated during the turnover of 2,2´,3-trihydroxydiphenyl-ether. Studies on this phenomenon have been presented (3.3).

The following experiment was set up to verify whether, by using whole cells overexpressing different extradiol dioxygenases, the 2,2´,3-trihydroxydiphenyl-ether produced by dioxygenation of dioxin could be, at least partially, subject to *meta*-cleavage. Resting-cells assays were set-up with *E. coli* DH10B expressing the module pMDK12 (corresponding to an OD of 5) mixed with *E. coli* BL21(DE3)[LysS] (pT7-22), *E. coli* BL21(DE3)[LysS] (pT7-23) or *E. coli* BL21(DE3)[LysS] (pTW7-5-RW), hyperexpressing the extradiol dioxgenases Edo2-2, Edo2-3, and DbfB respectively. Cells overexpressing the respective dioxygenases were added corresponding to an OD

of 5, such that the cell density in each system was approximately 10. Each mixed cell culture was tested for disappearance of the 2,2´,3-trihydroxydiphenyl-ether produced by *E. coli* DH10B (pMDK12) during incubation with 0.1 mM dioxin. Cells were precultured as described in 2.7.1.2. *E. coli* DH10B(pMDK12) cells of a final A_{600} of 5 and incubated with 0.1 mM dioxin were used as a control. The resting cell assay was conducted as described in 2.7.1.2. The reversed-phase HPLC analysis revealed that all three *meta*-cleavage dioxygenases are active on 2,2´,3-trihydroxydiphenyl-ether in the conditions used but exhibit different conversion rates. After an incubation period of 24 hours, all samples exhibited, as expected, quantitative depletion of dibenzo-*p*-dioxin (100 µM). However, quantitative accumulation of THB ether (> 90 µM) occurred only in the control. Slightly lower amounts of ether (approximately 80 µM) were observed in samples augmented with BL21(DE3)[LysS] (pTW7-5-RW), and significant lower amounts (approximately 25 and 15 µM, respectively) in samples augmented with *E. coli* BL21(DE3)(LysS)(pT7-23) or *E. coli* BL21(DE3)(LysS)(pT7-22).

The catechol produced by the *meta*-cleavage of 2,2´,3-trihydroxydiphenyl-ether and successive hydrolysis should be further cleaved by each of the three enzymes DbfB, Edo2-2 and Edo2-3. This explains the failure to detect by HPLC any catechol during the transformation of dibenzo-*p*-dioxin in above experiments. However, the catechol *meta*-cleavage product, 2-hydroxymuconic semialdehyde is yellow colored and thus can be detected in a spectrophotometric assay, when present in sufficient concentration.

Actually, in resting cell assay containing *E. coli* DH10B (pMDK12) mixed with *E. coli* BL21(DE3)[LysS] (pT7-22), or BL21(DE3)[LysS] (pT7-23), the appearance of an intense yellow color during the first hours indicated that catechol had been produced and was further cleaved by the extradiol dioxygenases Edo2-2 or Edo2-3. These enzymes can therefore be definitely considered key enzymes for dioxin metabolism in *Sphingomonas* RW1.

4 Discussion

In 1992, a *Sphingomonas* sp. strain named *Sphingomonas* sp. RW1, has been isolated from the river Elbe (Wittich *et al*., 1992) for its capability to grow on dioxin and dibenzofuran as sole carbon source. This strain was later on shown to be capable to also cometabolize several chlorinated congeners of dibenzofuran and dibenzo-*p*-dioxin. The pathways for the degradation of DD and DBF have been proposed (Wittich *et al*., 1992) and enzymes involved in the so-called upper pathway, i.e. the transformation of those compounds into salicylate and catechol, respectively have been purified and biochemically and genetically characterized. The first enzymatic activity involved in the catabolism of DD and DBF is driven by a dioxin dioxygenase. The enzyme has been purified and shown to be a multicomponent enzyme (Bunz and Cook, 1993). The enzymatic complex includes a reductase A2, a ferredoxin Fdx1 and a dioxygenase component, responsible for the initial angular dioxygenation of the two substrates. The second activity involved in the DD and DBF metabolism is driven by an extradiol or *meta*-cleavage dioxygenase. *Sphingomonas* sp. RW1 has been shown to contain at least three *meta*-cleavage enzymes (Happe *et al*., 1993) (Bunz *et al*., 1993) thus representing a class of isoenzymes, which all potentially could play a role in vivo in the metabolism of DDs and DBFs.

Beside the *meta*-cleavage dioxygenases, various other enzymes and the respective genes of the dioxin and dibenzofuran degradative pathway were shown to be present on the RW1 not only as a single gene, but the presence of isoenzymes or isogenes for various steps of the degradative pathway became evident. In particular two reductases, three ferredoxins and two hydrolases have been characterized (Armengaud *et al*., 1998; Armengaud and Timmis, 1997b; Bunz and Cook, 1993; Bunz *et al*., 1993). All structural genes have been found to be dispersed on the genome of RW1 and not organized in operons (Armengaud *et al*., 1998). Once the structural genes involved in the DD and DBF degradation pathways had been identified, it became possible to project the realization of a gene cassette carrying all genes necessary for the metabolism of DD and DBF into catechol or salicylate or of chlorinated DDs and DBFs into chlorocatechols or chlorosalicylates. The final intention is to use such a gene cassette to extend the degradation capabilities of microorganisms capable of mineralizing chlorocatechols or chlorosalicylates and by this realizing a bacterium able to completely

mineralize chlorinated DDs and DBFs. In this study we present the realization of this gene cassette besides further studies on the DD and DBF metabolism in RW1 and the characterization of a new extradiol dioxygenase observed in this organism, namely Edo3.

4.1 Genetic and biochemical characterization of Edo3 and Edo2 extradiol dioxygenases

One RW1 *meta*-cleavage dioxygenase, DbfB has been described by Happe to be capable of transformation of trihydroxyiphenyl and assumed to be involved in the degradation of dibenzofuran and dibenzo-*p*-dioxin. A second gene encoding a distinct *meta*-cleavage dioxygenase, named Edo2 was briefly described by J. Armengaud (Armengaud *et al*., 1998). In the current investigation we could show, that RW1 contains at least three *meta*-cleavage dioxygenases. The existence in some bacteria of multiple catabolic enzyme systems, such as ring-cleavage dioxygenases, is well established. As an example, *Rhodococcus globerulus* P6, capable of degrading biphenyl contains at least three (Asturias and Timmis, 1993), *Rhodococcus erythropolis* TA421 seven (Kosono *et al*., 1997), and the dibenzofuran degrading organism *Terrabacter* sp strain DPO360 (Schmid *et al*., 1997) at least three *meta*-cleavage dioxygenases. Such a diversity of isoenzymes in the same organism is often interpreted to reflect a broader spectrum of substrates that the microorganism can handle under disparate environmental conditions.

Actually, in *Rhodococcus* sp. I1 (Irvine *et al*., 2000), the inactivation of one of five extradiol dioxygenase genes abolished the capability of the strain to grow on isopropylbenzene and the inactivation of another one resulted in derivatives unable to grown on naphthalene. Obviously, the respective gene products have substrate specificities or their expression is regulated in a way that they are specifically involved in the degradation of only a subset of the growth substrates. Separate inactivation of two other *meta*-cleavage dioxygenase genes had no influence on the degradative phenotype, indicating that they either have a yet unkown or no obvious function, or that they can be substituted by one of the other genes encoding *meta*-cleavage enzymes. The fact that different *meta*-cleavage dioxygenases were involved in the degradation of a single substrate has already been reported by A. Schmid (Schmid *et al*., 1997). Three different dioxygenases were observed in cell extracts of dibenzofuran grown

Terrabacter sp. strain DPO 360, two of them exhibiting high activity against dihydroxy- or trihydroxybiphenyl, and thus assumed to be of importance for dibenzofuran degradation. Whereas the third enzyme was shown to exhibit high activity against catechol and thus is obviously of major importance for further degradation of intermediate catechol. A similar situation as in *Terrabacter* sp. strain DPO360 was observed in *Sphingomonas* sp. RW1. Inactivation of the *dbfB* gene resulted in derivatives growing slower with dibenzofuran. As, however, significant growth was still observed, it can be assumed that, together with the *dbfB* gene product, a second ring-cleavage dioxygenase is functioning during growth on dibenzofuran.

In the present report, two distinct *meta*-cleavage dioxygenases, Edo2 and Edo3, were genetically and biochemically characterized. The respective genes had originally been identified on two 10.5 Kb and 3300 bp DNA fragments from RW1, respectively. Immediately preceeding *edo3*, a gene with high similarities to a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase, and designated *H3*, was found, whose termination codon overlaps the starting codon of the Edo3 structural gene. Interestingly crude extracts of *E. coli* clones carrying both genes (*E.coli* pBl4) did not exhibit detectable *meta*-cleavage activity with the test substrate 2,3-dihydroxybiphenyl. This suggests that either H3 hydrolase further processes the 2,3-dihydroxybiphenyl ring cleavage product with such high activities that no intermediate accumulation of the yellow colored ring-cleavage product occurs, or that the transcription of *H3* gene prevents that one of *edo3*. Experiments analyzing this phenomenon are currently under investigation. The *edo3* gene was then amplified and cloned separately from the *H3* gene. This time *meta*-cleavage activity was observed with both the *E. coli* colonies on plate as well as with cell crude extracts or cell suspension. The sequence analysis of the *edo2* gene revealed the presence of three putative ATG starting codons. ATG2 directly follows ATG1 while ATG3 is localized 21 bp downstream of ATG2. A SD sequence was found in front of *edo2,* which seems to identify ATG2 as the most likely starting codon. Comparison of the subunit size of Edo2-2 (expressed with ATG2 as start codon) and Edo 2-3 (expressed with ATG3 as start codon) with that expressed from the original 3.3 Kb RW1 genomic insert subcloned in *E.coli* DH101, showed that, actually, the ATG2 start codon is used in this strain and thus, most probably, also in the original host RW1.

The protein sequences of Edo3, Edo2-2 and Edo2-3, directly deduced from the genetic one, have been aligned with those of several previously described extradiol-dioxygenases. Edo 3, Edo2-2 and Edo2-3 were found to be clustered with typical *meta*-cleavage enzymes specialized in bicyclic aromatic compounds. Interestingly the seven

amino acids at the N-terminus of Edo2-2 fall out of the protein alignment, as they do not fit the sequence of a typical extradiol dioxygenase. However, comparison of kinetic parameters between Edo2-2 and Edo2-3 revealed no significant differences, indicating those 7 amino acids to have no major influence on the substrate specificity.

In 1996 Lindsay D. Eltis and Jeffrey T. Bolin have performed an alignment of a selection of 35 extradiol dioxygenases protein sequences (Eltis and Bolin, 1996) and used the results to discuss the phylogenetic relationship among them and to revise the classification system for these enzymes. In that study DbfB clearly clustered in the same clade with 2 domains enzymes, preferring bicyclic compounds. In the present study the amino acid sequence data obtained from the DNA sequence of Edo3, Edo2-2 and Edo2-3 structural genes, has been newly aligned with those of related extradiol dioxygenases confirming the clustering of all RW1 extradiol dioxygenase with those of *meta*-cleavage dioxygenases preferring bicyclic compounds. All DbfB, Edo2-2, Edo2-3 and Edo3 preferred 2,3-dihydroxybiphenyl as a substrate over catechol, and can be assigned to class I.3 (following classification as in (Eltis and Bolin, 1996)) of C23O in agreement with the sequence data.

Despite the fact that all analyzed *meta*-cleavage enzymes preferred 2,3-dihydroxybiphenyl over monocyclic biphenyls, significant differences in the extend of this preference were observed. Whereas Edo2 (in both assessed variants) showed a reasonable activity against the monocyclic substrates catechol and 3-methylcatechol (specificity constants differed by only one order of magnitude), both Edo3 and DbfB (Happe *et al*., 1993) showed only negligible activity against those substrates (specificity constants differing by 3 to more than 4 orders of magnitude). In this aspect, Edo1 and 3 resembles the 2,3-dihydroxybiphenyldioxygenases purified from the dibenzofuran degrader *Terrabacter* sp. strain DPO360 (Schmid *et al*., 1997) whereas Edo 2 resembles those purified from the dibenzofuran degrader *Terrabacter* sp. strain DBF63 (Kasuga *et al*., 1997). All three dihydroxybiphenyl dioxygenases of RW1 showed a reasonable activity against trihydroxybiphenyl with specificity constants in the same order of magnitude as for dihydroxybiphenyl. Unfortunately, thus far only poor information is available on the kinetics of transformation of trihydroxybiphenyl by other dihydroxybiphenyl dioxygenases. However, the activity of such enzymes on trihydroxybiphenyl is not a unique feature restricted to a few specialized enzymes (Kasuga *et al*., 1997), (Asturias and Timmis, 1993) (Kohler *et al*., 1993). Kinetic properties have only been described for two extradiol dioxygenases of *Terrabacter* sp. strain DPO360 and for DbfB. All three enzymes showed a preference for dihydroxy-

over trihydroxybiphenyl with an extreme of bphC1 of *Terrabacter*, where the significant higher K_m value with trihydroxybiphenyl is the major reason for the 20-fold higher K_{cat}/K_m value for the dihydroxy- compared to the trihydroxybiphenyl. Edo 2 and Edo 3 of RW1 are thus the first enzymes with similar preferences for di- and trihydroxybiphenyl as substrates.

4.2 The 2,2,3-trihydroxybiphenyl ether metabolism

Despite of the fast inactivation of the extradiol dioxygenase in vitro, it was possible to characterize reaction products of this reaction by means of HPLC. In contrast to previous assumptions, the products of the reaction were characterized as catechol on the one hand and 2-pyrone-6-carboxylate on the other. 2-Hydroxymuconate was not observed as a product. Thus, the trihydroxybiphenylether ring cleavage product does not undergo a spontaneous hydrolysis as suggested by Wittich. In contrast, the ring-cleavage product underwent an intramolecular arrangement as shown during the transformation of 2,3-dihydroxybiphenylether by a *meta*-cleavage enzyme from *Pseudomonas cepacia* Et4 where phenol and 2-pyron-6-carboxylate were observed as ring-cleavage products (Pfeifer *et al*., 1993). It can be proposed that a nucleophilic attack of the hydroxyl oxygen of the opened ring on the ester carbon occurs which results in the displacement of phenol (in case of dihydroxybiphenyl ether transformation) or of catechol (in case of trihydroxybiphenyl ether transformation) and ring closure of the aliphatic residue to give the pyron. As 2-pyrone-6-carboxylate is not used as a growth substrate by RW1 and was not observed as a metabolite during growth on dibenzo-*p*-dioxin, it can be assumed, that in RW1 the ring-cleavage product undergoes a fast enzymatic hydrolysis by a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase thus preventing the spontaneous formation of the pyron. Even though it cannot be excluded thus far that, during growth of RW1 on dibenzo-*p*-dioxin, a hydrolase capable of transforming the pyrone is induced, the most probable postulate in light of those observations is that a hydrolase highly active on 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate is necessary not only for dibenzofuran but also for dibenzo-*p*-dioxin degradation.

Both Edo 2 as well as Edo3 *meta*-cleavage dioxygenases as well as DbfB were rapidly inactivated during the transformation of trihydroxybiphenyl ether. The sensitivity of *meta*-cleavage dioxygenases towards inactivation has already been described and can be

due to completely different mechanisms. Catechol 2,3-dioxygenses are usually very labile enzymes and are rapidly inactivated upon exposure to oxidizing agents such as oxygen (Nozaki *et al*., 1968). Inactivation of e.g. catechol 2,3-dioxygenase of *Pseudomonas putida* MT-2 also occurs during catalytic turnover when substrate analogues such as chloro- or alkylanalogues (Cerdan *et al*., 1995) are provided. As the inactivated enzyme could be reactivated by the incubation with $FeSO_4$, the authors assume that oxidation and/or removal of the ferrous ion in the catalytic center was responsible for the inactivation. Cerdan et al. (Cerdan *et al*., 1994) showed that the inhibition in case of the *Pseudomonas putida* mt-2 catechol 2,3-dioxygenase (class I.2.A) is not due to inactivation by the substrate itself nor due to the reaction products, but only occurs in the presence of oxygen plus substrate. This contrasts the observations of Klecka and Gibson (Klecka and Gibson, 1981) who showed that the catechol 2,3-dioxygenase of *Pseudomonas putida* F1 (class I.3.B) is inactivated by 3- and 4-chlorocatechol, even though those substrates are not transformed by the enzyme. Thus, in this case inactivation, which again was shown to be reversible upon addition of ferrous iron, does not need substrate turnover, but possibly binding of both oxygen and the substrate to the enzyme. Polissi and Harayama (Polissi and Harayama, 1993) first demonstrated that the inactivation of *Pseudomonas putida* mt-2 catechol dioxygenase can be overcome by the presence of the XylT gene product. Hugo et al. (Hugo *et al*., 1998) proved that the function of XylT, identified as a (2Fe-2S) ferredoxin, is the reduction of oxidized iron in the catalytic site and thus reactivation of the enzyme after being inactivated during substrate turnover.

Another mechanism has been described as suicide inactivation during the turnover of 3-chlorocatechol by the mt-2 derived catechol 2,3-dioxygenase (Bartels *et al*., 1984). It is assumed that the intermediate ring-cleavage product is highly reactive and binds irreversibly to the enzyme. This is assumed to be due to nucleophilic groups near the active site of the enzyme, which can attack the activated acyl-group carbon. Very recently, an enzyme has been described, which quickly produces 2-hydroxymuconate as a reaction product (Mars *et al*., 1997). It is assumed that, in this enzyme, the produced acylchloride undergoes a fast hydrolysis to produce 2-hydroxymuconate, thereby avoiding the irreversible inactivation of the enzyme, which otherwise occurs by the formation of covalent enzyme-product-conjugates.

In the present investigation, it was shown that the inactivation of Edo2 by trihydroxybiphenyl ether is not mediated by the substrate nor by any of the products, but occurs during the transformation reaction in the presence of oxygen. These results

exclude inactivation due to substrate mediated chelation of the iron from the active center of the enzyme. The experiments performed do not differentiate between inactivation due to oxidation of the ferrous iron in the catalytic center as described by Cerdan or inactivation due to the formation of a covalent enzyme-product-conjugate. The ring-cleavage product supposedly formed during the cleavage of trihydroxybiphenyl ether, is the 6-(2-hydroxyphenyl)ester of 2-hydroxymuconate which can be assumed to be very susceptible to nucleophilic attack and thus resembles the acylchloride formed during 3-chlorocatechol turnover by catechol 2,3-dioxygenases. Its susceptibility to nucleophilic attack is seen by the spontaneous rearrangement of the ester to form catechol and 2-pyron-6-carboxylate. However, F. Pfeifer et al. (Pfeifer *et al*., 1993) did not report on any observable inactivation of 2,3-dihydroxybiphenyl dioxygenase purified from the diphenylether degrading strain *Pseudomonas cepacia* Et4 during the turnover of 2,3-dihydroxybiphenylether.

During our experiments we observed turnover capacities of Edo 2 and Edo 3 of 40 - 50 nmol/U which are very similar to values reported for turnover capacities of those catechol 2,3-dioxygenases which are sensitive to inhibition during 3-chlorocatechol turnover (values of 60 - 220 nmol/unit of catechol 2,3-dioxygenase activity with catechol (Mars *et al*., 1999). Turnover numbers of 3-chlorocatechol by catechol 2,3-dioxygenases were determined by the group of Reineke (personal communication) and are about 100 substrate molecules transformed per enzyme molecule before complete inactivation of the enzyme occurred. The GJ31 enzyme resistant to that kind of inactivation showed turnover numbers of 11,000. Cerdan et al (Cerdan *et al*., 1994) reported a very fast inactivation of catechol 2,3-dioxygenase of *Pseudomonas putida* mt-2 during turnover of 4-ethylcatechol such that kinetic values could not be determined for this substrate. However, the turnover capacity was determined as a reasonable 6500 (amount of substrate molecules transformed before complete inactivation of the enzyme per enzyme molecule), whereas those of 3-methylcatechol and catechol were 210,000 and 1,400,000 respectively. Those inactivations were due to oxydation of the central ferrous iron and could be overcome by the presence of the XylT ferredoxin. It thus seems that inactivation of extradiol dioxygenases by the oxidation of the central ferrous iron depend on the structure of the substrate but seems to be much less effective than inactivation by the formation of covalent enzyme-product-conjugates. Even though Edo2 and 3 were not purified to homogeneity, their catalytic efficiencies could be estimated assuming their expression in *E. coli* to be in the same order of magnitude compared to DbfB. The thereby estimated Kcat values of 100 -

1000/sec are similar to those of various previously reported 2,3-dihydroxybiphenyl dioxygenases. This would correspond to turnover capacities in the order of magnitude of 100 -1000 molecules of trihydroxybiphenlether per enzyme molecule. Inactivation of Edo2 and 3 during trihydroxybiphenyl turnover is thus a very effective process. Cerdan et al. (Cerdan *et al*., 1994) calculated that a threshold of 18.000 in turnover number (before complete inactivation of an enzyme molecule) for a certain catechol derivative is necessary to support growth of *Pseudomonas putida* mt-2 on substituted aromatics. In case of growth of *Pseudomonas putida* GJ31 on chlorobenzene, a turnover number of 11.000 is enough to support growth. However, from these data it is evident that the simple presence of Edo2 or 3 during growth on dioxin would not be enough to support the observed growth. Assuming an inactivation mechanism as reported for 3-chlorocatechol turnover this means that the actual enzyme responsible for dibenzo-*p*-dioxin turnover remains to be discovered. Wittich et al. (Wiitich *et al*., 1992) in the first report describing growth of RW1 on dibenzo-*p*-dioxin indicated a reasonable trihydroxybiphenyl ether dependent of dibenzo-*p*-dioxin grown cells of 25 % that observed with trihydroxybiphenyl. In contrast, only very poor activity (3 % of that with trihydroxybiphenyl) in cell extracts was observed. This could hint on the presence of a highly unstable extradiol dioxygenase present in RW1. However, it cannot be excluded, that the trihydroxybiphenyl ether mediated inactivation of extradiol dioxygenases is due to an oxydation of catalytically active ferrous iron in the active center and that XylT analogues are present in RW1 allowing reactivation of extradiol dioxygenases.

4.3 Realisation of two modular compact genetic cassettes encoding respectively dibenzofuran and dioxin degradation upper pathways

The structural genes encoding for the enzymes involved in the dibenzofuran and dioxin degradation upper pathway were assembled in two compact modular genetic cassettes. As previously described, RW1 possesses several class of isoenzymes, specifically two reductases, three ferredoxins, three extradiol dioxygenases and three hydrolases. It was initially necessary to make a choice of the genes to be included in the two genetic modules depending on the encoded enzymes. The genes *dxnA1A2*, *redA2*, *fdx1*, codifying for the multicomponent dioxin dioxygenase were included in both gene cassettes. In particular *redA2* and *fdx1* genes were coamplified from the genomic libraray of RW1 in a single PCR product where *redA2* directly precedes *fdx1*.

The two compact modular genetic cassettes differ in the gene codifying for the extradiol dioxygenase. Previous studies conducted on DbfB (B. Happe, data not published) provided strong evidences that this enzyme could play the major role in the DBF degradation in RW1. In particular *dbfB* was knocked out in RW1. The metabolites produced after incubation of the knocked out strain in presence of DBF were analysed on HPLC. The HPLC analysis revealed accumulation in the culture medium of 2,2,3-trihydroxybiphenyl. For this reason, the first gene cassette, dedicated to dibenzofuran degradation involved the *dbfB* gene. The gene *edo 2-2* was choosen as the corresponding enzyme exhibits significant activity against trihydroxybiphenyl ether but also against catechol and 3-methylcatechol and thus a broader substrate specificity than Edo3. The gene *edo2-2*, complete of all putative ATG starting codons and the SD sequence was assembled in the DD dioxin cassette in place of *dbfB*. In both cassettes the last element is *dxnB*, the structural gene for the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase and capable of converting 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoic acid (data not shown). The resulting two cassettes were therefore *dxnA1A2_redA2-fdx1_dbfB_dxnB* also defined as dibenzofuran gene cassette, and *dxnA1A2_redA2-fdx1_edo2-2_dxnB*, defined as dioxin gene cassette. All elements of the two cassettes have been amplified by PCR by mean of primers introducing unique 8 base cutter restriction sites at their 3' and 5' ends and including for all genes the natural SD sequence. Each element in the gene cassette can therefore be easily exchanged with other ones for instance newly discovered genes of metabolic importance, as studies on RW1 strains develop. New elements could be added as well.

4.4 DD and DBF conversion studies

Experiments with both cassettes revealed that all enzymes relevant for the transformation of dibenzofuran into salicylate and of dibenzo-*p*-dioxin into catechol were expressed. This was evidenced for the initial dioxygenase by quantification of substrate transformation rates, for DbfB and Edo2-2 by quantification of 2,3-dihydroxybiphenyl dioxygenase activities in cell extracts and for DxnB by the formation of benzoate from 2,3-dihydroxybiphenyl or of salicylate from dibenzofuran. However, activity of the initial dioxygenase was very low in *E. coli* cells (less than 1 U/g protein). As expected, identical activity levels were observed for the initial dioxygenase in both modules. Activity of DbfB expressed from module I was significantly higher compared to that of Edo2-2 in module II (200U versus 20U/g protein when measured with dihydroxybiphenyl as substrate). This correlates with the higher activity of DbfB compared to Edo2-2 in the overexpression system (9.000 U versus 2700 U/g protein when measured with dihydroxybiphenyl as substrate) and could indicate that Edo2-2 actually has a lower catalytic activity compared to DbfB. Relatively low activity of DxnB was detected, but this activity was high enough to quantitatively convert 2-hydroxy-6-(2-hydroxyphenyl)-6-oxohexa-2,4-dienoate formed by the combined action of dioxin dioxygenase and DbfB into salicylate as expressed from module I. In this case, the substrate flow is limited by the activity of the initial dioxygenase and the ring-cleavage product is only produced at low rates. In contrast, DxnB activity was not high enough to prevent accumulation of 2-hydroxy-6-(2-hydroxyphenyl)-6-oxohexa-2,4-dienoate and thus spontaneous rearrangement into 3-(chroman-4-on-2-yl)-pyruvate when produced at a high rate by the direct action of DbfB on 2,2,3-trihydroxybiphenyl.

Whereas the gene cassette comprising *dbfB* was performing properly for the transformation of dibenzofuran into salicylate, the gene cassette comprising *edo2-2* was not. Despite the fact that the Edo2-2 activity with 2,3-dihydroxybiphenyl was only around 10 % of that of DbfB activity in *E.coli* cells used for transformation experiments, the respective activity with 2,3,3-trihydroxybiphenyl (ca. 10 U/g) on the first view should be sufficient for quantitative transformation of trihydroxybiphenyl produced from dibenzofuran (at a rate of 0.2–0.3 U/g). However, significant accumulation of trihydroxybiphenyl was observed during dibenzofuran transformation, indicating ring-cleavage to be rate limiting. The specific rates (U/g protein) of trihydroxybiphenyl transformation by whole cells of *E. coli* DH10B (pMDK19) and the

respective cell extracts differed by more than one order of magnitude and the low activities observed by whole cells were similar to those of dibenzofuran transformation and could indicate a bottleneck at this enzymatic step. However, the reasons for this observed differences remains to be elucidated. It should be noted, that Wittich et al. (Wittich *et al*.,1992) did not observe any significant difference (less than a factor of 2) between specific oxygen uptake rates by *Sphingomonas* sp. RW1 upon incubation with di- or trihydroxybiphenyl and the respective activities determined in cell extracts, indicating substrate uptake not to be rate limiting and activities in-situ to be similar to those measuresd in cell extracts.

Both gene cassettes enabled the respective *E. coli* derivatives to transform dibenzo-*p*-dioxin. However, only trihydroxybiphenyl ether was observed as transformation product. Such an outcome can be expected from the kinetic data of the extradiol dioxygenases. Assuming that an amount of Edo2-2, which can transform 1µmol of 2,3-dihydroxybiphenyl per minute, is capable of transforming 0.05 µmole of THBE, it can be calculated that *E. coli* (pMDK19) cells of an OD of 10 contain enough Edo2-2 to just transform a total of 1 µM trihydroxybiphenyl and thus 1% of the formed intermediate during the dibenzo-*p*-dioxin transformation experiments. Even though, higher activities of DbfB were observed in *E. coli* (pMDK12), the effectivity of this enzyme with THBE was shown to be lower than the effectivity of Edo2-2. The low effectivity of DbfB with THBE as substrate was confirmed by the experiments in which dibenzo-*p*-dioxin was transformed by *E. coli* cells expressing module I enzymes in the presence of *E. coli* cells overexpressing different extradiol dioxygenases. Less than 20 µM of THBE permanently released during dibenzo-*p*-dioxin turnover could be further transformed by *E. coli* BL21(DE3)(LysS)(pTW7-5RW) overexpressing DbfB present at an optical density of 5, a cell extract of which is capable of transforming 9000 µmole DHB/min g protein. Assuming further on OD of 5 to correspond to 0.5 g protein/l, the applied cell suspension should contain ca. 0.3 µM of DbfB. Thus the in-vivo turnover capacity of DbfB in *E. coli* for THBE is lower than 60 substrate molecules/enzyme molecule. In contrast to cells overexpressing DbfB, cells overexpressing Edo2-2 or 2-3 were capable of a significant further transformation (75–85 µM) of intermediary released trihydroxybiphenyl ether. The extend of further transformation was in the same order of magnitude as that calculated taking account the kinetic data determined in cell extracts for THBE transformation (70–190 µM). Thus, in *E. coli*, there are no auxiliary functions present which can overcome the observed inactivation of extradiol dioxygenases in-vivo or which can prevent to some extend such inactivation.

Consequently, RW1 either contains not yet discovered reactivation systems or not yet discovered other extradiol dioxygenases not subject to such an inactivation.

Recently, (Kasuga *et al*., 2001) characterized genes encoding an angular dioxygenase from the dibenzofuran degrader *Terrabacter* sp strain DBF63 and could show that the gene products of *dbfA1* and *A2*, together encoding the terminal oxygenase components, were capable in *E. coli* of angular dioxygenation of both dibenzofuran and dibenzo-*p*-dioxin. Similarly the *carA* genes of the carbazol degrader *Pseudomonas* sp. strain CA10 were described to transform dibenzo-*p*-dioxin and dibenzofuran by an angular attack (Sato *et al*., 1997) and even the biphenyl dioxygenase of the biphenyl degrader *Burkholderia* sp. strain LB400 was shown to catalyze angular dioxygenation of dibenzo-*p*-dioxin and dibenzofuran (Seeger *et al*., 2001). However, the presence in a strain of a dioxygenase enabling angular dioxygenation and a complete pathway for mineralization of dihydroxybiphenyl or analogues is not enough to enable a strain to mineralize dibenzofuran or dibenzo-*p*-dioxin. Moreover, even though being capable of dibenzofuran degradation, both *Terrabacter* sp. strain DBF63 or *Pseudomonas* sp. HH69 did not mineralize dibenzo-*p*-dioxin. This can be due to different reasons such as low transformation rates or inadequate or missing induction of the pathway enzymes by the substrates. Failure to grow on dibenzofuran can be due to the absence of salicylate hydroxylating activities in the strain analyzed. Harms described that, in strain HH69, the trihydroxybiphenyl ether undergoes an unexpected *ortho*-cleavage (Harms *et al*., 1990). They also reported that this strain does not grow on dibenzofuran in the presence of small amounts of dibenzo-*p*-dioxin and explained this by competitive inhibition of the *meta*-cleavage enzyme. In the light of the investigations in the current report, it can be proposed that the *meta*-cleavage enzyme in HH69 is fastly inactivated during trihydroxybiphenyl ether transformation, such that this compound is finally nearly quantitatively channeled into a simultaneously induced unproductive *ortho*-cleavage route. Kasuga et al. (Kasuga *et al*., 1997) reported that a dihydroxybiphenyl dioxygenase isolated from *Terrabacter* DBF63 is capable of transforming trihydroxybiphenyl ether into a yellow colored product. In contrast to their proposal, that the yellow coloration is due to the ring-cleavage product, it can be assumed that the coloration results from the cleavage of intermediary formed catechol as observed during the transformation of trihydroxybiphenyl ether by Edo2-2. However, the observed transformation does not give any indication of the efficiency of that reaction. Thus it can be proposed that in both HH69 and DBF63 the failure to grow on dibenzo-*p*-dioxin is due to a missing effective trihydroxybiphenyl ether transforming system.

Despite the fact that the developed gene cassettes function for dioxin only in the presence of a system overproducing extradiol dioxygenases, it can be concluded that the gene cassette comprising *dbfB* is functional for the degradation of dibenzofuran into salicylate. It can be thus a useful tool to rationally assemble, in future, complete degradative pathways for dibenzofuran, and, after elucidation of the transformation capacity for chlorinated dibenzofurans, to assemble organisms capable to mineralize chlorodibenzofurans. It was recently shown (Arfmann *et al*., 1997), that a coculture of *Sphingomonas* sp. RW1, transforming 4-chlorodibenzofuran into 3-chlorosalicylate and *Burkholderia* sp. RWS is capable to mineralize 4-chlorodibenzofuran. Taking into account the biochemistry of transformation as well as transformation rates of chlorodiobenzofurans (Wilkes *et al*., 1996) it can be reasoned that micorooragnisms capable to mineralize chloro- as well as some dichlorodibenzofurans can be constructed using the gene module constructed here under a suitable regulatory system.

5 Summary

Extradiol dioxygenases have crucial roles in the degradation of various aromatic compounds, including the degradation of dibenzo-*p*-dioxin and dibenzofuran by *Sphingomonas* sp. RW, where such enzymes are responsible for the cleavage of the pathway intermediates 2,2´,3-trihydroxybiphenyl (THB) and 2,2´,3-trihydroxybiphenyl ether (THBE). In this study we showed that at least three different extradiol dioxygenases are encoded in the RW1 genome, namely DbfB (Edo1), Edo2 and Edo3 which extends our knowledge on the genetic puzzle of catabolic genes in RW1.

The Edo3 structural gene, 800 bp long, has been found to be preceded by a ORF, encoding a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase, H3. The *edo3* ATG start codon overlaps the *H3* stop codon. Activity of Edo3 was observed when *edo3* was cloned separately from *H3*. The sequence analysis of the *edo2* (892 bp) gene revealed the presence of three putative ATG start codons and ATG2 was identified as the most likely one used in RW1 during the transcription of *edo2*.

From the alignment of the amino acid sequences, Edo2 and Edo3 (like DbfB) were shown to cluster with extradiol dioxygenases specialized in transforming bicyclic aromatics. The respective substrate preferences were confirmed in kinetic experiments. However, Edo 2 and Edo 3 of RW1 are the first enzymes described with similar preferences for di- and trihydroxybiphenyl as substrates.

All three dioxygenases underwent rapid inactivation during transformation of THBE. Despite rapid inactivation, catechol and 2-pyrone-6-carboxylate could be identified as reaction products of ring-cleavage and enzymatic tests to follow and kinetically characterize THBE transformation were developed. In this study it was shown that the inactivation is not mediated by the substrate nor by any of the reaction product but dependent on the presence of oxygen and substrate turnover. The inactivation was very effective, especially for DbfB where turnover numbers lower than 60 were determined but less pronounced for Edo3 and especially Edo2.

The structural genes encoding for the enzymes involved in the dibenzofuran and dioxin degradation upper pathway were then assembled into two compact modular genetic cassettes with the intent to use them for the realization of genetically engineered microorganisms able to completely mineralize chlorinated dibenzofurans and dioxins. Those cassettes were constructed in a fashion enabling the rational exchange of a

certain gene by another one encoding for a protein of similar function, thus allowing the analysis of the function of various members of a family of genes in the degradation of a certain substrate. Based on the kinetic properties of their gene products, *dbfB* (dibenzofuran gene cassette) and *edo2* (dibenzo-*p*-dioxin degradation) were included in those cassettes. It was evidenced that all enzymes relevant for the transformation of dibenzofuran into salicylate and of dibenzo-*p*-dioxin into catechol were expressed from both cassettes.

The gene cassette comprising *dbfB* was performing properly as evidenced by quantitative transformation of dibenzofuran into salicylate without accumulation of intermediates or side-products. Both gene cassettes transformed dibenzo-*p*-dioxin as far as THBE only, due to in-vivo inactivation of extradiol dioxygenases. By the presence of cells overexpressing different extradiol dioxygenases, it could be verified that Edo2 is suited, however imperfectly, for the further transformation of THBE.

6 Abbreviations:

A	Adenine
Ap	Ampicillin
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BPH	Biphenyl
BSA	Bovine serum albumin
bp	Base pairs
C	Cytosine
CBP	Chlorinated biphenyl
Cm	Chloramphenicol
DBF	Dibenzofuran
dbfB	THB-dioxygenase gene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
edo2	Gene for extradiol dioxygenase
edo3	Gene for extradiol dioxygenase
EDTA	Ethylenediaminetetraacetic acid
FPLC	Fast Protein Liquid Chromatography
G	Guanine
g	Gram
H3	Gene for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase
h	Hour
HOPDA	2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl-thio-ß-D-galactopyranoside
Kb	Kilobase
K_{cat}	Maximal turnover number
K_d	Dissociation constant
kDa	Kilodalton

Km	Kanamycin
K_m	Michaelis constant
l	Liter
M	Molar
m	milli-
µ	micro-
min	minute
n	nano-
nt	nucleotide
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
RBS	Ribosome binding site
rpm	Rounds per minute
S	Substrate
SD	Shine Dalgarno
SDS	Sodium dodecyl sulphate
sp.	Species
T	Thymine
Tab.	Table
TAE	Tris-acetate/EDTA
TEMED	N,N,N,N-Tetramethylethylenediamine
THB	2,2',3-trihydroxybiphenyl
THBE	2,2',3-trihydroxybiphenyl ether
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
UV	Ultraviolet light
V	Volt
v	volume
V_{max}	Maximal enzymatic activity at substrate saturation
w	Weight
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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